

ROLES OF SELENOPROTEIN M (SELENOM) IN HYPOTHALAMIC
LEPTIN SIGNALING AND CALCIUM REGULATION

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ABSTRACT

Selenium is an essential trace element that is critical for human health. The biological effects of selenium are largely mediated by selenoproteins, a unique class of proteins that contain selenocysteine (Sec) as an integral part of their polypeptide chain. Selenoprotein M (SELENOM) is an ER-resident thiol-disulfide oxidoreductase that is most abundant in the brain. It contains a thioredoxin-like domain (cysteine-X-X-selenocysteine) that catalyzes thiol-disulfide exchange. It has been reported that SELENOM has neuroprotective functions and is implicated in regulation of Ca^{2+} homeostasis. Our group previously published that *Selenom*^{-/-} mice display increased weight gain, elevated white adipose tissue deposition, and impaired hypothalamic leptin sensitivity compared to wild-type mice, suggesting a role for SELENOM in energy homeostasis. Therefore, we performed a series of studies using *in vivo* and *in vitro* models to investigate the specific influence of SELENOM on hypothalamic leptin signaling, ER stress, and Ca^{2+} signaling. The evidence gathered in this study revealed that SELENOM promotes hypothalamic leptin signaling, inhibits ER stress, and regulates Ca^{2+} influx. To further delineate the underlying mechanism, we then assessed the subcellular localization and binding partners of SELENOM. Using co-immunoprecipitation and liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) to screen for SELENOM-binding partners, we discovered and verified two proteins, microtubule associated protein 6 (MAP6) and choline-phosphate cytidyltransferase 1 alpha (PCYT1A). In addition, we found that SELENOM localizes not only in ER but also in mitochondria-associated ER-membranes (MAMs), suggesting a potential role of SELENOM in regulation of Ca^{2+} signaling. Finally, we performed microarray analysis using both *Selenom*^{-/-} hypothalamic tissue and mHypoE-44 cells to identify the genes and signaling pathways most affected by SELENOM. Our results revealed 11 genes that were significantly altered by SELENOM deficiency, including thioredoxin-interacting protein (TXNIP), a negative regulator of the thioredoxin (TXN) system. SELENOM deficiency also

significantly reduced TXN activity in both hypothalamic tissue and mHypoE-44 cells. In summary, our studies reveal that SELENOM promotes hypothalamic leptin signaling, potentially due to its functions in ER stress, Ca^{2+} signaling, and the hypothalamic TXN system. In addition, we further determined that SELENOM localizes in MAMs and interacts with MAP6 and PCYT1A, suggesting an important role in MAMs-modulated Ca^{2+} homeostasis.

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Agouti-related protein (AgRP), Adenosine monophosphate-activated protein kinase (AMPK), Atonal homolog 1 (Atoh1), Arcuate nucleus (ARC), Activating transcription factor-6 (ATF6), Alstrom syndrome protein 1 (Alms1), Apolipoprotein E receptor 2 (ApoER2), Apoptosis signal-regulating kinase 1 (ASK-1), Brown adipose tissue (BAT), Blood-brain barrier (BBB), Basic helix-loop-helix (bHLH), Breast Cancer 1 (Brca1), Binding immunoglobulin protein (BiP), Cysteine (Cys), CCAAT-enhancer-binding protein homologous protein (CHOP), CCAAT/enhancer binding protein delta (Cebpd), Cyclin Dependent Kinase Inhibitor 1A (Cdkn1a), Cerebrospinal fluid (CSF), Central nervous system (CNS), Differentially Expressed Genes (DEGs), Endoplasmic reticulum (ER), Extracellular signal-regulated kinase (ERK), Eukaryotic initiation factor 4a3 (eIF4A3), Epidemiology of Vascular Ageing (EVA), Endoplasmic reticulum resident protein 44 (ERp44), Familial Alzheimer's disease (FAD), Fatty acid-CoA ligase 4 (FACL-4), Glutathione peroxidases (GPxs), glucose-regulated protein 75 (Grp75), High fat diet (HFD), Hepatocellular carcinoma (HCC), Iodothyronine deiodinase (Dio), Immunohistochemistry (IHC), Inositol requiring enzyme 1 (IRE1), Inositol-1, 4, 5-triphosphate receptor (IP3R), Interleukin 1 (IL-1), Janus kinase 2 (JAK2), Leptin (Ob), Leptin receptor (ObR), Lipocalin-2 (Lcn2), Liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS), Mitochondria associated ER-membranes (MAMs), Median eminence (ME), Microtubule associated protein 6 (MAP6), Methionine (Met), Mammalian target of rapamycin (mTOR), Neuropeptide Y (NPY), Nicotinamide adenine dinucleotide phosphate (NADPH), Paraventricular nuclei (PVN), Choline-phosphate cytidylyltransferase 1 alpha (PCYT1A), Peroxisome Proliferator-Activated

Receptor Gamma, Coactivator 1 Alpha (PGC1 α), Protein kinase RNA-like ER kinase (PERK), Protein disulphide isomerase (PDI), Proopiomelanocortin (POMC), Presenilin-2 (PS2), Protein tyrosine phosphatase 1B (PTP1B), Pituitary adenylate cyclase activating polypeptide (PACAP), Phosphatidylcholine (PC), Phosphatase and tensin homolog (PTEN), Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), Reactive oxygen species (ROS), Recommended Dietary Allowance (RDA), Ribosomal protein L30 (RPL30), Ribosomal L1 Domain Containing 1 (Rsl1d1), Ryanodine receptors (RyRs), Selenium (Se), Selenocysteine (Sec), Selenomethionine (SeMet), Selenocysteyl-tRNA (Sec-tRNA^{[Ser]^{Sec}}), Sec insertion sequence (SECIS), SECIS binding protein 2 (SBP2), Sec-specific elongation factor (eEFSec), SECIS binding protein 2 like protein (SECISBP2L), Selenoprotein P (SELENOP), Selenoprotein M (SELENOM), Selenoprotein H (SELENOH), Selenoprotein O (SELENOO), Selenoprotein T (SELENOT), Selenoprotein K (SELENOK), Selenoprotein W (SELENOW), Selenoprotein V (SELENOV), Selenophosphate Synthase 2 (SPS2), Signal transducer and activator of transcription 3 (Stat3), Thapsigargin (Tg), Thioredoxin (TXN), Thioredoxin reductase (TXNRD), Thioredoxin interacting protein (TXNIP), Type2 diabetes mellitus (T2D), Unfolded protein response (UPR), Unsaturated free fatty acids (FFA), Voltage-dependent anion channels type 1 (VDAC1), Tolerable Upper Intake Level (UL), X-box binding protein-1 (XBP1s), 15 kDa selenoprotein (SELENOF)

CHAPTER 1. INTRODUCTION

Selenium

Selenium (Se) is an essential trace element that is critical for human health. It was first discovered and named by a Swedish chemist, Jöns Jacob Berzelius, as a by-product from sulfuric acid production in 1817. In the environment, Se can be found naturally in water, soil, and a variety of foods. Foods rich in Se include Brazil nuts, seafood, grain, cereals, organ meats, some green vegetables, and shiitake mushrooms. Se concentration in plants depends on the Se content in the surrounding soil, which varies tremendously between areas worldwide, ranging from deficiency (close to zero) to 1250 mg/kg [1-4]. Regions with especially high Se levels in the soil include Enshi County in China, North and South Dakota in the USA, and some parts of Ireland and Colombia. In Enshi County, plants will uptake and accumulate high levels of Se from the soil, leading to a potential risk of selenosis for local residents [5, 6]. There are also areas with Se deficient soil, such as Congo, New Zealand, Finland, some regions of Arizona and Montana in the USA, a wide belt extending from northeast to southwest parts of China, and central Serbia [7-9]. Other factors that contribute to plant Se uptake from soil include the relative concentrations of other elements (iron, sulphur, and aluminum) and soil pH [10, 11]. Se deficiency is implicated in Keshan disease, Kashin-Beck disease, and myxedematous endemic cretinism, which are characterized by congestive cardiomyopathy, osteoarthropathy, and mental retardation, respectively [12-15]. In addition, Se deficiency also contributes to decreased thyroid function, metabolism disorders, immune dysfunction, several neurological conditions, increased cancer risk, and male infertility [2, 3]. In the environment, Se is present as inorganic (selenite, selenate, selenide, elemental Se) and organic chemical forms, mainly including selenomethionine (SeMet) and selenocysteine (Sec) [16, 17]. Both organic and inorganic forms of Se can be absorbed in small intestine and utilized in various body tissues [18]. These Se metabolites are taken up and absorbed primarily by enterocytes with varying efficiency, depending on the presence of other trace elements and the specific biochemical forms of Se [19-23]. However, the mechanisms by which these Se metabolites are absorbed and processed vary. Selenite is believed to passively diffuse across the plasma membrane while selenate is absorbed via an sodium-mediated carrier mechanism and reduced to selenite before being further metabolized [20, 24]. The organic form of Se, SeMet, is

absorbed by methionine (Met) transporters and incorporated into proteins at the methionine positions in direct proportion to the relative content of Met [25]. Together with the existing intracellular Se pool, these Se sources will be metabolized via different pathways and eventually become selenide, which is the direct precursor for selenocysteine (Sec) biosynthesis.

A growing interest has emerged over past decades in the correlation between Se intake and human health, which was initiated with ground breaking experiments in rodents [26]. Researchers found that the range between therapeutic and toxic doses of Se is very narrow and that the effects of Se supplementation depend on multiple factors, including ingested forms and dose of Se [27-30]. In 1989, the World Health Organization suggested a recommended daily allowance (RDA) of Se, 55 µg for women and 70 µg for men. However, different countries have established their own recommendations of Se intake. Since 2000, the US RDA for Se has been changed to 55 µg per day for both women and men, whereas Tolerable Upper Intake Level (UL) is 400 µg per day by the National Academies of Science's Institution of Medicine. In some European countries, such as the UK, Denmark, Norway, and Germany, the RDA for Se ranges from 50 µg to 75 µg per day. Different from other countries, Japan established a much lower RDA at 25 µg and 30 µg per day for women and men, respectively. These recommendations were established based on the minimum Se level required to obtain optimal plasma glutathione peroxidase (GPx) activity [31, 32]. However, recent studies indicate that selenoprotein P (SELENOP) may be a better indicator for Se status since higher Se intake is required for optimal SELENOP expression [33]. Human studies provide evidence of a U-shaped association between Se intake and various health risks [34, 35].

Selenoproteins

Se carries out important biological functions through its incorporation into selenoproteins in the form of the 21st amino acid, Sec, which was first discovered in the 1970s [36]. In humans, there are 25 distinct selenoproteins that are widely distributed in various organs. These proteins include the glutathione peroxidases (GPxs), thioredoxin reductases (TXNRDs), iodothyronine deiodinases (Dios), selenophosphate synthase 2 (SPS2), and several additional Sec-containing proteins. Other than the aforementioned selenoenzymes, the functions of most of the members of this class of proteins are not

well understood, but are currently under investigation. A list of the 25 human selenoproteins and known functions is presented in Table1.

Selenoprotein Synthesis

To understand the distinct features and structures of selenoproteins, first, we need to understand the mechanisms by which Sec is incorporated into selenoproteins. Sec incorporation into proteins is essential for many fundamental functions in humans [37]. The UGA codon, normally a stop codon, codes for Sec in the coding regions of selenoprotein genes [38]. Cis-acting factors and trans-acting factors bring a selenocysteyl-tRNA (Sec-tRNA^{[Ser]Sec}) to the ribosome. This Sec-tRNA^{[Ser]Sec} recognizes the selenocysteine-insertion codon (UGA) in the coding region of selenoprotein mRNAs. However, Sec-tRNA^{[Ser]Sec} alone is not sufficient to decode UGA codons as Sec. A specific RNA stem-loop structure in the 3' untranslated region (3' UTR) of selenoprotein mRNAs, termed a Sec insertion sequence (SECIS) element, is also required. The SECIS element was first discovered and defined in iodothyronine deiodinase 1 (Dio1) [39]. The selenoprotein translation process also requires SECIS binding protein 2 (SBP2) and Sec-specific elongation factor (eEFSec) [40-43]. However, some fundamental questions of this distinct process still remain unclear, particularly the functions of other factors, such as the SECIS binding protein 2 like protein (SECISBP2L) [41, 44], eukaryotic initiation factor 4a3 (eIF4A3) [45], and ribosomal protein L30 (RPL30) [46].

Selenoprotein Families

Most selenoproteins display antioxidant functions through the amino acid, Sec. For example, GPx and TXNRD family members are well characterized as antioxidant enzymes that regulate redox homeostasis and protect organisms from damage caused by reactive oxygen species (ROS).

GPxs were the first identified mammalian proteins that incorporate Se as Sec into the active site [47]. The GPx family reduces hydrogen peroxide and organic hydroperoxides, functioning in a protective role against oxidative stress. In humans, there are seven GPx proteins of which five are selenoproteins. These Sec-containing GPxs (GPx1-4, GPx6) are distributed in different tissues and utilize different substrates for peroxide removal, such as hydrogen peroxide, fatty acid hydroperoxides, phospholipid

hydroperoxides, and cholesterol hydroperoxides. GPxs are essential components in the human antioxidant system and their loss has been linked to Keshan disease, metabolic syndrome, and different types of cancer. Interestingly, *GPx1*^{-/-} and *GPx2*^{-/-} mice are healthy and fertile under normal conditions, whereas *GPx4*^{-/-} mice are embryonic lethal [48, 49]. GPx4 directly reduces phospholipid hydroperoxides and cholesterol hydroperoxides. Moreover, it is also involved in male fertility and sperm maturation as a redox-active enzyme in spermatids.

TXNRD, along with thioredoxin (TXN) and nicotinamide adenine dinucleotide phosphate (NADPH), comprises the TXN system, which contributes to redox homeostasis and is essential for life. There are three mammalian TXNRDs: TXNRD 1 localized to the cytosol, TXNRD 2 localized to mitochondria, and testis-specific TXNRD 3. They catalyze the reduction of TXN using electrons transferred from NADPH. The TXN system plays a critical role in development, as deletion of TXN, TXNRD1, or TXNRD2 in mice results in embryonic death [50-53].

The Dio family consists of three different Sec-containing oxidoreductases, Dio1, Dio2 and Dio3, which regulate the activity of thyroid hormones. Dio1 and Dio2 catalyze the activation of thyroxine (T4) into 3, 5, 3-triiodothyronine (T3), whereas Dio3 inactivates T4 into reverse T3, a metabolically inactive form. Therefore, Dios are implicated in diseases with impaired thyroid hormone metabolism, such as hyperthyroidism and hypothyroidism [54].

Selenoprotein P (SELENOP) is a secreted protein that contains two domains, an N-terminal domain with one Sec in a UXXC redox motif and a C-terminal domain containing 9 Secs. The high selenium content of SELENOP indicates that it is involved in selenium transport or storage [55]. The liver produces the highest amounts of SELENOP, and exports the protein to other organs, such as the brain, testis, and kidney. Apolipoprotein E receptor 2 (ApoER2) and megalin bind to SELENOP and mediate its uptake into testis and kidney, respectively [56, 57].

Selenoproteins in Metabolic Disorders

Selenoproteins have a wide range of effects documented in literature, including cancer prevention, preventing heart diseases, antioxidant properties, regulating immune function, thyroid hormone activation, and insulin sensitivity regulation. Animal and human studies have revealed complex correlations between

selenoproteins and metabolic disorders. A study in a high-fat diet/STZ-induced T2D rat model has revealed that Se supplementation (180-500µg/Kg/Day) can reduce blood glucose and cholesterol level [58]. In clinical studies, a positive correlation of low Se level with T2D incidence has been reported in senior French men and samples from Spain [59, 60]. However, these disparate findings lack consensus. Other randomized studies have revealed a positive correlation between Se intake and metabolism disorders [61-63]. The roles of individual selenoproteins in metabolism may explain some of the controversial findings in these human studies.

GPx1, or cytosolic GPx, is expressed ubiquitously. Transgenic mice overexpressing GPx1 develop insulin resistance, hyperglycemia, elevated body weight, hyperinsulinemia, and obesity [64], indicating that high levels of GPx1 may interfere with insulin function. Furthermore, the hyperinsulinemia was not corrected when these mice were challenged with a Se deficient diet [65]. This finding in GPx1-overexpressing mice is consistent with results from another study in which elevated GPx1 was associated with mild insulin resistance in pregnant women [66, 67]. The expression of GPx1 is known to be sensitive to Se intake. Rats with Se supplementation had high levels of GPx1 in the liver and showed increased protein tyrosine phosphatase 1B (PTP1B) activities, a known antagonist of insulin signaling [68]. Moreover, *GPx1*^{-/-} mice were protected from high-fat-diet-induced insulin resistance due to increased ROS, leading to the oxidation and inhibition of phosphatase and tensin homolog (PTEN), which terminates phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K) signaling. With GPx1 deficiency, excessive ROS inactivated pancreatic protein tyrosine phosphatase N2 (PTPN2), promoted signal transducer and activator of transcription 1 (Stat1) signaling, and inhibited pancreatic and duodenal homeobox-1 (Pdx1) expression. In turn, this signaling cascade inhibited β cell insulin secretion and, consequently, improved pancreatic insulin signaling and suppressed hepatic glucose production [69]. Taken together, the function of GPx1 in metabolic disorders may depend on many factors, including tissue-specific GPx1 expression and different development states of T2D.

Another selenoprotein involved in the development of T2D is selenoprotein P (SELENOP), a liver derived secretory protein. Misu et al. reported that increased hepatic SELENOP mRNA levels positively correlated with insulin resistance in humans [70]. Moreover, administration of SELENOP impaired insulin signaling and glucose homeostasis in both hepatocytes and myocytes, whereas SELENOP deficiency

improved insulin sensitivity and glucose tolerance. This metabolic function of SELENOP is mediated via inactivation of adenosine monophosphate-activated protein kinase (AMPK), a positive regulator of insulin synthesis and secretion in β cells. The same group also demonstrated that SELENOP deficiency protects mice against obesity, insulin resistance, and hypoadiponectinemia resulting from a high fat, high sucrose diet [71]. In clinical studies, SELENOP has also been shown to have a strong correlation with glucose homeostasis. Serum SELENOP levels were found higher in T2D and prediabetic Korean and Japanese patients compared to people with normal glucose tolerance [66] [72]. In addition, in obese and overweight subjects in this study, SELENOP levels were higher than those observed in lean subjects, suggesting that SELENOP influences carbohydrate metabolism.

In addition to GPx1 and SELENOP, other selenoproteins are also implicated in metabolic syndrome including GPx4, Dio2, and SELENOT. *Dio2*^{-/-} mouse model has disruptions in energy metabolism and insulin resistance [73]. Studies in vivo and in vitro have revealed that SELENOT is critical for pancreatic β cell function and pituitary adenylate cyclase activating polypeptide (PACAP)-induced insulin secretion [74, 75]. In a recent study, hypothalamic GPx4 levels were found to decrease in response to a high fat and high sucrose diet [76]. Conditionally knocking out GPx4 in orexigenic hypothalamic neurons expressing agouti-related protein (AgRP) results in increased weight gain and adiposity in male mice. The multifaceted correlation between selenoproteins and metabolic disorders is still a focus of interest to obtain a clear understanding of Se in metabolic homeostasis.

Selenoprotein M (SELENOM)

Discovery and Structure

SELENOM was first identified in *silico* in 2002 by Korotkov et al. as a 0.7kb cDNA sequence that coded for a new protein in mammalian EST databases [77]. The authors reported that this gene has a 145-amino acid open reading frame that begins with an AUG codon in a favorable Kozak context and that Sec is encoded by the 48th codon. In addition, they also determined that the human analogue of SELENOM localizes to chromosome 22 (22q12.2), spanning 3kb with 5 exons and 4 introns. The first 23 amino acids act as a signal peptide that promotes SELENOM translocation to the ER [77]. The remaining parts of SELENOM contain a central two-layer α/β sandwich, consisting of three α -helices and four β -

strands, and an ER retention signal tetrapeptide in the C-terminus after helix $\alpha 3$. SELENOM, along with several other selenoproteins including SELENOF, SELENOH, SELENOO, SELENOT, SELENOV, and SELENOW, contains a TXN-like domain, CXXU (U: Sec), at the active site which catalyzes thiol-disulfide exchange. Structure studies of SELENOM using 3D computer modeling determined that the C-terminal region of SELENOM is highly flexible and does not assume a regular secondary structure [78, 79]. Ferguson et al. suggested that the flexible C-terminal region is a potential binding site of SELENOM for protein substrates [78].

Expression

The first analysis of SELENOM distribution in mammalian tissues revealed that SELENOM mRNA is expressed in the brain, heart, stomach, lung, kidney, and thyroid [77]. Among these tissues, SELENOM is most abundant in the brain, with low levels in other organs. Utilizing data from the Allen Brain Atlas, Zhang et al. conducted a comparative analysis of selenoprotein gene expression in the mouse brain. It was documented that GPx4, SELENOK, SELENOM, SELENOP, SELENOW, and SELENOF have the highest mRNA expression in the brain [80]. SELENOM mRNA levels are found highest in the hippocampus, specifically the CA1/CA2 regions, the dentate gyrus regions, the main olfactory bulb, and the cerebellar cortex [80]. Zhang et al. also performed immunohistochemistry (IHC) and Western blotting to confirm protein expression of SELENOM in different brain regions. IHC results showed SELENOM expression in the hippocampus and the cerebellar cortex, whereas Western blot analysis revealed high levels of SELENOM in whole brain samples and the cerebellar cortex. We conducted Western blot analysis on different mouse brain regions and the results revealed that SELENOM protein expression was highest in the cerebellum and olfactory bulb. In addition, the hippocampus and cerebral cortex had the lowest protein levels of SELENOM, while the hypothalamus and the brain stem had intermediate levels. To confirm these findings, IHC was performed, showing that SELENOM is highly expressed in the cerebellar cortex, the arcuate (ARC) and paraventricular nuclei of the hypothalamus, the medial septum, the reticular thalamus, the red nucleus, the ventral tegmental area, various auditory brainstem nuclei (cochlear nucleus, lateral lemniscus, superior olive), and the CA2/CA3 region of the hippocampus. Western blot results of various mouse organs further determined that the pancreas and the pituitary also

express high levels of SELENOM, comparable to that of the brain [81]. Interestingly, microarray analysis of human cochlea and vestibule revealed that SELENOM was detected as one of 52 genes whose signal intensities were 10-fold higher in the cochlea and vestibule than in a mixture of other tissues [82].

SELENOM expression can also be regulated by a variety of factors, including various transcription factors, Se supplementation, and leptin treatment [83-85]. Potential AP1 transcription factor binding sites were found in SELENOM by sequencing the putative transcription initiation regions in human selenoproteins [86]. SELENOM has been documented to be upregulated by X-box binding protein 1 (XBP1s), an ER-stress responsive transcriptional activator [83]. Moreover, the SELENOM mRNA levels were reduced by fasting and restored by leptin treatment in the hypothalamic paraventricular nucleus (PVN) [84]. Microarray analyses of the Atoh1-specific population of the dorsal neural tube relative to the neighboring Neurog1-specific population identified SELENOM as one of the transcripts regulated by atonal homolog 1 (Atoh1). Atoh1 is a basic helix-loop-helix (bHLH) transcription factor that is critical for the formation of different proprioceptive neuronal subtypes [87]. Another important influence on SELENOM expression is Se supplementation. A recent study examined the effect of dietary Se deficiency and age on Se status and selenoprotein mRNA expression in male and female mice [88]. SELENOM mRNA levels were consistently downregulated by dietary selenium deficiency in the kidney and liver of both sexes as well as in the heart of the female mice. These results are consistent with the findings from Sunde et al. who reported that SELENOM expression was moderately influenced by Se status in the kidney and liver, but not as significantly as that of GPx1, SELENOH, and SELENOW [89]. In addition, SELENOM expression, as well as that of GPx1, SELENOH, and SELENOW, was found to be regulated by Se status in mouse colon [90]. Further, levels of SELENOM mRNA and protein in chicken brain were reduced by dietary Se deficiency [91].

Neuroprotective Role

Both animal models and cell culture studies have demonstrated that SELENOM has neuroprotective properties and contributes to intracellular Ca^{2+} regulation. Utilizing a transgenic mouse model of Alzheimer's disease expressing a mutant human presenilin-2 (PS2) gene, Hwang et al. found that these mice had significantly suppressed expression of SELENOM in the brain [92]. Subsequent studies to

determine the neuroprotective function of SELENOM were performed using a new transgenic rat model that overexpressed human SELENOM. These transgenic rats had increased levels of antioxidant enzymes such as GPx and superoxide dismutase (SOD) in various tissues [93]. Further exploring the mechanisms in the antioxidant role of SELENOM revealed that SELENOM overexpression as well as Se supplementation significantly activated the extracellular signal-regulated kinase (ERK) signaling pathway, inhibited α/γ -secretase activity, and reduced the phosphorylation of Tau protein [94].

Studies *in vitro* also contributed evidence that SELENOM has neuroprotective properties and is involved in intracellular Ca^{2+} homeostasis [95]. SELENOM overexpression in hippocampal HT22 cells was associated with reduced superoxide production and apoptotic cell death while SELENOM knockdown resulted in a high apoptotic cell death rate. Moreover, overexpression of SELENOM decreased intracellular Ca^{2+} flux induced by hydrogen peroxide, whereas knockdown of SELENOM increased the cytosolic Ca^{2+} levels. The disturbance of Ca^{2+} homeostasis is a major feature of many central nervous system (CNS) disorders, including Alzheimer's disease. These results indicated that SELENOM regulates Ca^{2+} levels and may protect the brain from oxidative stress [95, 96].

Characterization of the *Selenom*^{-/-} Mouse

The transgenic mouse model with a targeted deletion of the SELENOM gene was generated and characterized [81]. These mice were raised on standard laboratory chow with adequate Se (~0.25ppm) and compared to wild-type mice on a C57BL/6J background to identify potential functions of this protein [81]. *Selenom*^{-/-} mice displayed normal brain morphology, anxiety-like behavior, motor coordination, and cognition. Whereas no cognitive deficits were observed, these mice exhibited increased weight gain and elevated white adipose tissue deposition. In addition, serum leptin levels were significantly higher in *Selenom*^{-/-} male mice and this did not correspond with differences in the glucose tolerance or hepatic insulin signaling. In a subsequent study, SELENOM was found co-expressed with the leptin receptor in neurons of the ARC hypothalamus. Compared to wild-type controls, *Selenom*^{-/-} mice were found to have diminished leptin sensitivity in the ARC hypothalamus, indicating leptin resistance. Food consumption was then assessed and observed to be comparable between *Selenom*^{-/-} mice and controls. Therefore, the increase in body weight of the transgenic mice is likely due to diminished energy expenditure and

reduced movement. Further studies performed on both genotypes fed a Se-deficient diet (Se concentration < 0.01ppm) confirmed the main findings as *Selenom*^{-/-} mice fed with Se-deficient diet were observed to exhibit less movement, increased weight gain, and elevated fat deposition. At the same time, glucose tolerance was found to be comparable between wild-type and *Selenom*^{-/-} mice when fed either a normal or Se-deficient diet. Additionally, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis was found in the *Selenom*^{-/-} mice. Leptin is known to promote activation of the HPA axis [97]. Combining the findings of increased leptin level and the altered base-line corticosterone levels, the results indicate that the relationship between leptin signaling and the HPA axis may be dysregulated by SELENOM deficiency. Taken together, these studies provide compelling evidence that SELENOM plays a critical role in energy metabolism [81]. However, whether the observed impairments in leptin signaling were the primary cause or a secondary response of obesity was not determined.

Central hypothesis

SELENOM promotes hypothalamic leptin signaling and regulates cytosolic Ca²⁺ levels.

Specific aims

Aim 1: Assess the specific roles of SELENOM in leptin signaling, ER stress, and cytosolic Ca²⁺ regulation. Our *Selenom*^{-/-} mouse model was observed to have increased weight gain and develop leptin resistance, suggesting that SELENOM may play an important role in leptin signaling. In addition, SELENOM was found co-expressed with the leptin receptor in the arcuate hypothalamus, a region involved in energy metabolism and leptin signaling [81]. However, it is still unclear whether this leptin resistance is the cause or a secondary complication of the obesity. One predominant underlying cause of leptin resistance is ER stress. Moreover, SELENOM was implicated in intracellular Ca²⁺ regulation [95]. Therefore, we hypothesize that SELENOM may promote leptin signaling by inhibiting ER stress and regulating cytosolic Ca²⁺ levels. In this aim, we will perform experiments *in vitro* and *in vivo* to assess the influence of SELENOM upon leptin signaling, ER stress, and Ca²⁺ signaling.

Aim 2: Determine the subcellular localization and interaction partners of SELENOM. SELENOM is an ER-resident protein and implicated in intracellular Ca^{2+} regulation. We hypothesize that SELENOM may reside at the ER/mitochondria interface to regulate Ca^{2+} trafficking between ER and mitochondria, and in turn, regulate mitochondrial metabolism. In addition, we speculate that SELENOM may also physically interact with proteins involved in energy metabolism and Ca^{2+} regulation. For this aim, we will determine the subcellular localization of SELENOM in mouse brain tissue using a well-established subcellular fractionation protocol for isolation of mitochondria associated ER-membranes (MAMs). In further experiments, we will identify SELENOM interaction partners using co-immunoprecipitation and LC-MS/MS, respectively.

Aim 3: Determine genes and pathways significantly affected by SELENOM deletion in hypothalamus. *Selenom*^{-/-} mouse model was observed to have significant weight gain, suggesting that SELENOM may play an important role in energy metabolism. In addition, SELENOM is highly expressed in the hypothalamus, a region critically involved in energy metabolism and leptin signaling. For this aim, we will conduct microarray analysis using both hypothalamic tissue and mHypoE-44 cells to identify genes and signaling pathways mostly affected by SELENOM deficiency.

Expected outcomes

Aim 1: SELENOM will promote leptin signaling, inhibits ER stress, and regulates cytosolic Ca^{2+} levels. We anticipate that leptin treatment will increase SELENOM expression in hypothalamic tissues and that SELENOM deficiency will significantly impair the leptin signaling *in vivo*. In addition, SELENOM deficiency is anticipated to promote ER stress and cytosolic Ca^{2+} level induced by thapsigargin. Finally, leptin is anticipated to decrease intracellular Ca^{2+} levels in control cells and this effect will be abrogated by knocking down or knocking out SELENOM.

Aim 2: SELENOM will interact with proteins involved in Ca^{2+} homeostasis and energy metabolism in the ER, and potentially the MAMs. We anticipate that SELENOM may localize to MAMs and interact with proteins involved in energy metabolism and Ca^{2+} regulation. Some thioredoxin family members and ER-resident selenoproteins, including SELENOK and SELENON, have already been indicated in

interactions with IP3 receptors and SERCA pumps [98-101]. Therefore, we anticipate that SELENOM may interact with IP3 receptors, ryanodine receptors, or SERCA pumps. Further identification of SELENOM interaction partners will be performed using co-immunoprecipitation.

Aim 3: SELENOM deletion will affect genes which are involved in energy metabolism and Ca²⁺ homeostasis. SELENOM have been implicated in cytosolic Ca²⁺ regulation, leptin signaling, and ER stress. Therefore, we anticipate that genes/pathways involved in leptin signaling, calcium regulation, and ER stress response may be altered by SELENOM deficiency. Moreover, SELENOM is known as a member of TXN superfamily. We also anticipate that genes/pathways involved in antioxidant activities will be affected when SELENOM is deficient.

Table 1. The Human Selenoproteome and Functions of Selenoproteins.

Selenoprotein	Abbreviation	Functions
Glutathione peroxidases ^{1,2,3,4,6}	GPx 1–4,6	Reduce lipid hydroperoxides and free hydrogen peroxide
Iodothyronine Deiodinase ^{1,2,3}	Dio ^{1,2,3}	Thyroid hormone metabolism
Thioredoxin Reducase ^{1,2,3}	TXNRD ^{1,2,3}	Reduce TXN
Selenophosphate Synthase 2	SPS2	Sec synthesis
Methionine Sulfoxide Reductase B1	MsrB1	Catalyze the reduction of methionine-R-sulfoxide to methionine
15 kDa-selenoprotein	SELENOF	Protein folding
Selenoprotein H	SELENOH	Genome maintenance and redox sensing
Selenoprotein I	SELENOI	Lipid metabolism, protein folding
Selenoprotein K	SELENOK	Ca ²⁺ flux in immune cells, ER-associated degradation
Selenoprotein M	SELENOM	Ca ²⁺ homeostasis, Energy metabolism
Selenoprotein N	SELENON	Ca ²⁺ homeostasis
Selenoprotein O	SELENOO	Unknown
Selenoprotein P	SELENOP	Transport of Se
Selenoprotein S	SELENOS	ER-associated degradation
Selenoprotein T	SELENOT	Ca ²⁺ mobilization and neuroendocrine secretion
Selenoprotein V	SELENOV	Unknown
Selenoprotein W	SELENOW	Muscle growth and differentiation

CHAPTER 2. ROLES OF SELENOM IN HYPOTHALAMIC ER STRESS, LEPTIN SIGNALING, AND CALCIUM REGULATION

Abstract

Selenoprotein M (SELENOM) is an endoplasmic reticulum (ER)-resident thiol-disulfide oxidoreductase that is highly expressed in hypothalamic regions involved in leptin signaling and energy metabolism. Moreover, *Selenom*^{-/-} mice exhibit adult-onset obesity, elevated serum leptin levels, and diminished leptin sensitivity in hypothalamus, indicating leptin resistance. However, whether SELENOM directly influences hypothalamic leptin signaling is yet unclear. One predominant underlying cause of leptin resistance is ER stress. Herein, we performed a series of studies using *in vivo* and *in vitro* models to investigate the specific influence of SELENOM on hypothalamic leptin signaling, ER stress, and Ca²⁺ signaling. The evidence gathered in this study revealed that SELENOM promotes leptin signaling by inhibiting ER stress and regulating Ca²⁺ influx in the hypothalamus.

Introduction

The incidence of obesity and type 2 diabetes is growing rapidly and has become an indisputable threat to human health worldwide. Selenoproteins are a distinct class of proteins that have been increasingly implicated in metabolic disorders [64, 70, 73, 81]. These proteins contain selenocysteine (Sec) as an integral part of their polypeptide chain and have protective roles against oxidative stress. SELENOM is an ER-resident thiol-disulfide oxidoreductase that is highly expressed in the brain, the hypothalamus in particular [77, 80]. As a member of TXN superfamily, SELENOM has an active site consisting of a Sec-containing TXN-like domain (CXXU) which catalyzes thiol-disulfide exchange reactions. Previous studies conducted *in vitro* suggested that SELENOM has neuroprotective functions and regulates Ca²⁺ homeostasis [95]. Moreover, in our *Selenom*^{-/-} mouse model, we found increased weight gain, elevated white tissue deposition, higher serum leptin levels, and diminished leptin sensitivity, which suggest that

SELENOM plays an important role in leptin signaling and energy metabolism [81]. However, the mechanism by which SELENOM regulates leptin signaling still remains unknown.

Leptin (Ob), a hormone derived from adipose tissue, plays a critical role in the regulation of food intake and energy expenditure [102]. The median eminence (ME), which serves as a route for the entry of the circulating leptin into the cerebrospinal fluid (CSF), contains a cluster of permeable fenestrated vessels [103]. Peripheral leptin first reaches the ME via these fenestrated vessels and binds to the leptin receptors expressed by tanycytes, which are specialized hypothalamic glia-like cells. They form a barrier between the circulating blood and the 3rd ventricle of the brain, and therefore mediate the transport of leptin into the CSF [103]. Once across the ME, leptin binds to transmembrane leptin receptors which are particularly concentrated in the arcuate nucleus of the hypothalamus (ARC) [104, 105]. Here leptin acts on two main populations of neurons expressing the leptin receptor, proopiomelanocortin (POMC)-expressing anorexigenic neurons and agouti-related peptide (AgRP) / neuropeptide Y (NPY) / inhibitory γ -aminobutyric acid (GABA) -expressing orexigenic neurons. AgRP neurons are well known to positively regulate feeding behavior, as brain-specific administration of either AgRP or NPY increases food intake [106, 107]. Both AgRP/NPY and POMC neurons project to the melanocortin-4 receptor (MC4R) -expressing neurons in the PVN. AgRP is known as a hypothalamus-specific antagonist of MC4R while α -melanocyte stimulating hormone (α -MSH), a POMC-derived peptide, activates the MC4R [108]. Therefore, AgRP neurons can inversely regulate the melanocortin pathway to reduce satiety and promote food intake [107, 109]. Several studies have reported that leptin stimulation on AgRP/NPY neurons can decrease cytosolic Ca^{2+} levels and inhibit food intake *in vivo* [110-113]. Binding with leptin receptor stimulates leptin receptor dimerization and activates the autophosphorylation of Janus kinase 2 (JAK2) [114, 115]. JAK2 then phosphorylates two key tyrosine residues on leptin receptors, including Y1138, which in turn, activates signal transducer and activator of transcription 3 (Stat3) signaling [116]. This leptin signaling pathway is inhibited by two adaptor molecules, suppressor of cytokine signaling 3 (SOCS3) and protein tyrosine phosphatase 1B (PTP1B). SOCS3 gene expression is upregulated by leptin-induced pStat3 and in turn, mediates the feedback inhibition via binding to leptin receptor to block the leptin pathway [117-119]. Another mediator, PTP1B, dephosphorylates JAK2 and subsequently diminishes downstream leptin signaling [120] (Figure 2-1). In the obese state, Stat3 phosphorylation is poorly stimulated by leptin [121]. Observations from animal models

indicate that obesity results from reduced leptin signaling in the brain. This contrasts with increased circulating levels of leptin, suggesting that leptin resistance in the brain is a major reason for the failure of leptin to reduce feeding behavior and stimulate energy metabolism in obesity. Thus, leptin resistance is a major obstacle in treatment of obesity (Fig. 2-2) [122].

Although the precise mechanistic basis of leptin resistance remains elusive, some biological defects have been proposed to be underlying causes [123]. One predominant underlying cause of leptin resistance is ER stress, which leads to altered intracellular Ca^{2+} signaling and occurs in response to accumulation of misfolded proteins and/or Ca^{2+} depletion within the ER [116, 124]. ER stress can activate a variety of cellular processes via signaling pathways termed the unfolded protein response (UPR) in order to restore ER functions. UPR is initiated by three different ER-localized proteins: protein kinase-like ER kinase (PERK), inositol requiring enzyme 1 ($\text{IRE1}\alpha$), and activating transcription factor-6 (ATF6) [125]. In an unstressed state, these three proteins are inactive via association with glucose-regulated protein 78 (GRP78), the most abundant chaperone in the ER. In response to accumulation of unfolded proteins, GRP78 dissociates from these three ER stress sensors and translocates to the ER lumen for protein folding. This dissociation changes the conformation of PERK, $\text{IRE1}\alpha$, and ATF6, leading to their activation (Fig.2-3) [126, 127]. Activation of the $\text{IRE1}\alpha$ pathway induces splicing of X-box binding protein 1 (XBP1s) mRNA [128] and subsequent transcription of molecular chaperones and various genes involved in protein folding, including SELENOM [83]. It has also been documented that activation of $\text{IRE1}\alpha$ -mediated spliced XBP1 in proopiomelanocortin (POMC) neurons protects against diet-induced obesity and improves leptin and insulin sensitivity [129]. This evidence suggests that SELENOM may be a positive regulator of leptin signaling by regulating sensitivity to ER stress and intracellular Ca^{2+} signaling.

Hence, we performed experiments *in vitro* and *in vivo* to assess the influence of SELENOM upon leptin signaling, ER stress, and Ca^{2+} signaling. In these studies, we found that SELENOM is a leptin-responsive gene that promotes hypothalamic leptin signaling by regulating ER stress signaling and Ca^{2+} homeostasis.

Materials and Methods

Chemicals and antibodies

Thapsigargin and ionomycin were purchased from Sigma. Primary antibodies used in these experiments were rabbit anti-phospho-Stat3 Tyr705 (1:1000; Cell Signaling, 9145), rabbit anti-Stat3 (1:1000; Cell Signaling, 8768), mouse anti-SELENOM (1:500; Santa Cruz, sc-514952), rabbit anti-phospho-PERK Thr980 (1:1000; Cell Signaling, 3179), mouse anti-CHOP (1:1000; Cell Signaling, 2895), and mouse anti- β -Actin(1:5000; Cell Signaling, 3700). Species specific secondary antibodies were purchased from LI-COR. Recombinant mouse leptin (Ob) was purchased from R&D Systems and diluted to a stock concentration of 1 mg/ml in 20 mM Tris-HCl, pH 8.0.

Animals and leptin challenge

Male wild-type and *Selenom*^{-/-} mice based on a C57BL/6N background were born, bred, and raised for 10 weeks in the University of Hawai'i Vivarium (N \geq 5 for each group). The mice were fasted overnight, given an intraperitoneal injection of leptin (1 μ g/g body weight), and sacrificed 1hr later with CO₂ asphyxiation. Mouse whole brains were removed promptly and immersed in 0.32M ice-cold sucrose for 5-10 minutes. The brain was then sliced with the ventral side up in a mouse brain slicer matrix with slice intervals of 1 mm. Two coronal cuts were made anteriorly at the level of the optic chiasm and posteriorly 3 mm to the first cut. Under a dissection microscope, this slice was then oriented coronally and the hypothalamus was dissected as a trapezoid consisting of the roof of the 3rd ventricle dorsally and the fornix laterally, and snap-frozen for later experiments.

Cell culture

The hypothalamic cell line, mHypoE-44, was purchased from Cellutions and grown in Dulbecco's Modified Eagle medium (DMEM) (Sigma) with 10% FBS (Invitrogen) (32nM) and 1% penicillin in a humidified incubator with 5% CO₂ at 37°C. This cell line has a neuronal morphology and is derived and immortalized from mouse embryonic day 17 hypothalamic primary cultures by retroviral transfer of SV40 T-Ag. In addition, mHypoE-44 cells are known to express NPY, AgRP, and leptin receptor [130, 131].

Cells were serum-starved for 4 hours before ER stress induction and leptin treatment. ER stress was induced by incubating cells in the presence of 1 μ M Tg (Sigma) for 4 hours and leptin treatment was carried out by challenging with 100nM leptin for 45 minutes.

Overexpression of SELENOM and leptin receptors

A SELENOM cDNA was synthesized and subcloned into pSelExpress1 vector (gift from Vadim Gladyshev's Lab) by GenScript. This SELENOM overexpression plasmid was transfected into mHypoE-44 cells using Lipofectamine 2000 (Thermo Fisher Scientific). Transfected cells were used for experiments 48 hours later (Fig. 2-4C). mHypoE-44 cells express the leptin receptor (ObR). However, we found that leptin responses were much stronger (Fig. 2-4D) if cells were transfected with an exogenous plasmid expressing the ObR (pObR) (a gift from Christian Bjorbaek's Lab). Therefore, for all leptin stimulation studies, a similar procedure was used to transfect a leptin receptor plasmid.

Generation of stable hypothalamic cell line with SELENOM shRNA

Constructs of short hairpin RNAs (shRNA) for mouse SELENOM and a non-target control were subcloned into the pTRIPZ vectors with the Tet-On® inducible system with RFP (Thermo Scientific). Hypothalamic cells at 70%-80% confluency were transfected with pTRIPZ vectors with SELENOM or non-target control shRNA using Lipofectamine 2000 (Thermo Fisher Scientific). Transfected cells were selected by media containing 1.5 μ g/ml puromycin (Sigma) for the next 7 days. Following the selection with puromycin, doxycycline (Clontech Labs) (final concentration=2 μ g/ml) was added to the culture media to 48hr prior to experiments to induce TurboRFP/shRNAmir expression. RFP expression was examined by microscope to evaluate transfection efficiency (Fig. 2-4A).

CRISPR/Cas9-mediated SELENOM deletion in mHypoE-44 cells

CRISPR/Cas9 technology was utilized for CRISPR/Cas9-mediated deletion of SELENOM expression in mHypoE-44 cells. Cells were first transfected with pSpCas9 (Addgene) plasmids expressing GFP. After selection by fluorescence-activated cell sorting (FACS) (BD Bioscience) to isolate cells successfully expressing Cas9, cells were allowed to recover for an additional 24hr period and then transfected with the guide RNA targeting exon 1 of the SELENOM gene (sequence: CCGGATTGGAACCGTCTTCG, IDT) and

universal tracrRNA (IDT). Following another 24hr recovery period, cells were subjected to a second round of sorting for GFP-positive cells and then cultured in 96-well plates at a density of 1 cell/well. We verified the knockout efficacy by Western blot assay and PCR after colonies formed (Fig. 2-4B). Primers for verification of SELENOM that flank exon 1 were purchased from IDT (Forward: 5'- GATTTGGGTGGGATGTCAGT-3', reverse: 5'- TCATGCGGCTGGGAAATAA -3'). DNA Sequencing data shows that there is a deletion of 'TCTTCGAG' from the 342nd to the 349th basepair of the SELENOM cDNA sequence.

Western blot

After different treatments, samples were prepared by sonication using CelLytic MT buffer (Sigma) with protease/phosphatase inhibitors (Cell signaling) and cleared by centrifugation. Samples consisting of 40 µg of protein were resolved on a denaturing 4-20% SDS-PAGE gel (Bio-Rad) and transferred to polyvinylidene fluoride membranes by electroblotting. The membranes were then blocked in Odyssey blocking buffer in PBS (LI-COR) for 30 minutes and incubated with specific primary antibodies at 4°C overnight. Blots were incubated with species-specific IR-dye coupled secondary antibodies (LI-COR) at room temperature for half an hour in the dark the next day. The signals were detected and analyzed by Image Studio Version 5.2 (LI-COR Odyssey).

Calcium imaging

Changes in intracellular Ca²⁺ level were determined by the Ca²⁺ indicator Fluo-4/AM (Invitrogen) using confocal imaging (Nikon). An appropriate amount of cells were seeded to sterile cover slips one day before imaging. These cells were loaded with 5 µM Fluo-4/AM (Invitrogen) for 45 minutes at 37°C. Cells were then washed three times with live cell imaging solution (Invitrogen) before imaging using a Nikon Diaphot microscope attached to a Nikon PCM 2000 in the Kaka'ako Health Sciences Microscopy and Imaging Core Facility. Images were recorded using excitation at 488 nm, emission at 515–565 nm, and 40× oil lenses. Responses were recorded as a time course, consisting of confocal images taken at 5 sec/interval for a total of either 150 or 300 sec. Tg (2 µM) or leptin (100 nM) was added at 30 sec. Ionomycin (1 µg/ml) (Sigma) was used as a positive control to elicit maximal Ca²⁺ responses. At least twelve random cells were selected for each experiment. Fluo-4 signals associated with individual cells were assessed via ImageJ software as

fluorescence was converted to signal intensity and normalized to baseline (F/F_0) for quantification purposes. Data are presented for at least three independent experiments.

Statistical analysis

Data were analyzed using unpaired Student's t-tests (for 2 groups) or two-way ANOVA (for > 2 groups) with Bonferroni post-test analysis (GraphPad Prism Software 7.0). Results were represented as means \pm SEM of at least three independent experiments. P values were calculated with the appropriate statistical tests using GraphPad Prism software 7.0. A significant difference is considered to be present at $*p < 0.05$.

Results

SELENOM protein level is increased by leptin treatment. To investigate the effect of leptin on SELENOM expression and determine whether hypothalamic leptin resistance happens prior to the development of obesity in *Selenom*^{-/-} mice, we conducted a leptin challenge on 10-week-old wild-type and *Selenom*^{-/-} mice with comparable body weights. Hypothalamic tissue was isolated and then subjected to Western blot analysis for SELENOM expression and leptin-induced Stat3 activation. Results demonstrated that SELENOM protein level was increased by leptin treatment in wild-type hypothalamic tissues (Fig. 2-5A, B). Western blot analyses also revealed that Stat3 phosphorylation induced by leptin treatment was significantly decreased in the *Selenom*^{-/-} group compared to the wild-type group (Fig. 2-5 A, C). Taken together, these results suggest that SELENOM is a leptin-responsive gene and promotes hypothalamic leptin signaling.

SELENOM promotes leptin signaling in mHypoE-44 cell line. To further define the function of SELENOM in leptin signaling, we performed a set of experiments using the mHypoE-44 cell line, an immortalized cell line derived from embryonic mouse hypothalamic neurons that expresses AGRP and NPY [132]. A construct encoding SELENOM short hairpin RNA (shRNA) was used to generate a stably transfected mHypoE-44 cell line where SELENOM expression was knocked down approximately 75% upon

induction with doxycycline. Meanwhile, we also generated a *Selenom*^{-/-} mHypoE-44 cell line with CRISPR/Cas9 editing. SELENOM knockdown and *Selenom*^{-/-} mHypoE-44 cells were treated with leptin at 100 nM for 45 min and assessed for Stat3 signaling by Western blot. As expected, leptin treatment increased pStat3 levels and this induction was impaired by SELENOM deficiency (Fig. 2-6). To further confirm the role of SELENOM in leptin signaling, we overexpressed SELENOM in mHypoE-44 cells. Cells overexpressing SELENOM had notably higher levels of pStat3 compared to the control cells with leptin treatment (Fig. 2-7). Interestingly, total Stat3 levels were also elevated by leptin treatment in cells overexpressing SELENOM. Taken together, these results verify that SELENOM is a positive regulator of leptin signaling.

SELENOM regulates the cytosolic Ca²⁺ level change induced by leptin. Our previous findings revealed that SELENOM has neuroprotective function and regulates Ca²⁺ homeostasis under conditions of oxidative stress [95]. In addition, it has been also demonstrated that leptin treatment induces hyperpolarization and leads to a reduction in cytosolic Ca²⁺ levels in AGRP neurons [110, 133]. As mentioned previously, mHypoE-44 cells have been reported to express AGRP and may display some features characteristic of AGRP neurons [132]. Subsequent observation by live imaging under confocal fluorescent microscopy showed that leptin treatment led to a corresponding reduction of baseline cytosolic Ca²⁺ in the control group, and this effect was abrogated in SELENOM shRNA-expressing cells (Fig. 2-10 A, B). Similar results were found in our *Selenom*^{-/-} cell line, as leptin failed to reduce cytosolic Ca²⁺ levels (Fig. 2-10 C, D). The results reveal that SELENOM is potentially a critical key in Ca²⁺ regulation in terms of leptin signaling.

Increased ER stress in SELENOM-deficient hypothalamic cells. ER stress is notable for its causative role in leptin resistance [134]. There are several pharmacological reagents which can induce ER stress through several different mechanisms, such as tunicamycin, thapsigargin (Tg), and Brefeldin A. In the present study, Tg was used to induce ER stress. It is a specific inhibitor of the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase (SERCA) which can induce a decrease in ER Ca²⁺

levels and cause the accumulation of unfolded proteins in the ER. To study the specific role of SELENOM in ER stress, mHypoE-44 cells were first transfected with SELENOM shRNA and then subjected to Tg (1 μ M) treatment for 4 hours. The cells were then prepared for Western blot analysis. Upon ER stress, PERK pathways activate CCAAT/enhancer-binding protein homologous protein (CHOP), a pro-apoptotic transcriptional factor [135]. Significant higher levels of pPERK and CHOP levels were observed in response to Tg treatment in cells where SELENOM was knocked down compared to control cells (Fig. 2-8A). Overexpression of SELENOM had the opposite effect (Fig. 2-9), which supports the conclusion that SELENOM regulates ER stress pathways in hypothalamic cells.

SELENOM regulates the cytosolic Ca^{2+} level change induced by Tg. To further test the relationship between SELENOM and the regulation of intracellular Ca^{2+} signaling, we carried out live imaging experiments of hypothalamic cells with Tg treatment. There was a more significant increase of intracellular Ca^{2+} level in cells with SELENOM knockdown in response to Tg compared to control cells (Fig. 2-11A, B). The increase in Ca^{2+} influx was potentially from the culture media or intracellular Ca^{2+} stores. We further repeated this experiment in Ca^{2+} -free extracellular media. Intracellular Ca^{2+} responses displayed the same pattern but the difference relative to the control group was smaller (Fig. 2-11C, D). These results provide evidence that increased cytosolic Ca^{2+} in response to ER stress may be from intracellular stores such as the ER. Similar effects were found in *Selenom*^{-/-} cells (Fig. 2-12). Parallel to our previous Western blot results, these results suggest that SELENOM plays an important role in regulating Ca^{2+} store signaling.

Discussion

With the rise in prevalence of obesity and type 2 diabetes, much effort has been made to investigate the mechanisms that regulate body weight and energy homeostasis. The hypothalamus is the central site that governs food intake and energy expenditure. Within the hypothalamus, neurons in the ARC nucleus monitor energy status by sensing hormones and nutrients in the circulating blood, such as leptin. Leptin

can initiate several signaling cascades of which JAK2-Stat3 signaling is the most prominent. The notion that hypothalamic selenoproteins are critical in regulating leptin signaling was verified recently using conditional knockout of Sec-tRNA (Trsp) mouse models with rat-insulin-promoter-driven-cre (RIP-Cre) and specific pancreatic- β cell-cre (Ins1-Cre), respectively[4]. This Trsp^{RIP} KO model disrupted Trsp expression in certain hypothalamic cells and pancreatic β cells. The authors evaluated the metabolic phenotypes in these two mouse models when challenged with a high fat diet. Trsp^{RIP} KO mice exhibited severe disruptions in glucose tolerance, insulin resistance, and leptin resistance, whereas the Trsp^{Ins1} KO mice did not display the same metabolic abnormalities. Moreover, upon leptin treatment, the Trsp^{RIP} KO mice displayed a much lower hypothalamic pStat3 level than the control group, indicating that hypothalamic leptin resistance occurs upon disruption of selenoprotein synthesis in hypothalamus. In our study, we demonstrated that the intraperitoneal injection of leptin increased levels of SELENOM in wild-type mice, which indicates that SELENOM is a leptin-responsive gene. Moreover, compared to wild-type mice, *Selenom*^{-/-} mice displayed decreased hypothalamic Stat3 activation in response to leptin, indicative of leptin resistance. Studies *in vitro* further verified that SELENOM is involved in promoting leptin signaling. These findings indicate that hypothalamic SELENOM plays an important role in leptin signaling.

To date, established causes of leptin resistance include inflammation, autophagy and ER stress. The ER is a dynamic organelle that is involved in protein folding, fatty acid and phospholipid synthesis, and Ca²⁺ homeostasis. Cellular disturbances that cause the accumulation of misfolded proteins trigger activation of the UPR. The tight link of ER stress and obesity was first reported by Ozcan et al [136]. Since then, accumulating studies have provided further evidence that hypothalamic ER stress promotes leptin resistance [116]. In diet-induced obesity models (DIO), UPR signaling pathways, including PERK, IRE1 α , and ATF6, have been found overactivated, in conjunction with impaired leptin signaling. In addition, mice receiving an intracerebroventricular injection of ER stress inducers developed leptin resistance along with an increase food intake and body weight gain [116]. Similar results were also found *in vitro* that leptin-induced pStat3 signaling was significantly reduced when treated with ER stress inducers [129]. Alternatively, when ER stress was ameliorated by ER stress inhibitors, such as tauroursodeoxycholic acid and 4-phenylbutyrate, ER stress makers were downregulated and leptin

sensitivity was rescued in DIO mice [116, 124, 137]. Our findings provide novel evidence revealing that SELENOM is a modulator of leptin signaling in the hypothalamus while inhibiting the ER stress response.

Within the cell, the ER is the major storage site for intracellular Ca^{2+} , whereas cytosolic Ca^{2+} concentrations are kept at low levels via the actions of many transporters, pumps and exchangers. ER stress can be triggered by abnormalities in Ca^{2+} signaling. Severe ER stress promotes Ca^{2+} efflux between the ER and mitochondria, which causes modification in mitochondria membrane potential and permeability, leading to apoptosis [138]. In our study, we hypothesized that the impaired leptin sensitivity and elevated ER stress response observed in *Selenom*^{-/-} samples may largely related to disrupted Ca^{2+} signaling. Our Ca^{2+} imaging data revealed that SELENOM deficiency mitigated the reduction of intracellular Ca^{2+} in response to leptin and promoted the Ca^{2+} release from the intracellular Ca^{2+} store under the treatment of ER stress inducer. However, the precise mechanisms still need to be explored. As mentioned above, SELENOM levels were significantly reduced in transgenic mice overexpressing the human mutant PS2 gene that causes familial Alzheimer's disease (FAD) [92]. PS2 is documented to play a critical role in calcium homeostasis via altering the expression or sensitivity of the ryanodine receptor (RyR), inositol 1,4,5-trisphosphate receptor (IP3R), and SERCA pump [139, 140]. Moreover, presenilins are enriched in ER mitochondria-associated membranes and alterations in PS2 expression affects Ca^{2+} shuttling between ER and mitochondria [141, 142]. There is accumulating evidence indicating that Ca^{2+} homeostasis is significantly affected by the redox status of the surrounding environment. Disturbances in redox balance and ER stress increase cytosolic Ca^{2+} levels and enhance the release of Ca^{2+} from ER via different channels. Redox modification of RyRs leads to ER Ca^{2+} leak and ROS was found to specifically sensitize IP3Rs through a thiol group(s) to promote Ca^{2+} efflux [143-145]. It was also interesting to find that selenium-supplementation reduced the IP3R-operated Ca^{2+} release from intracellular store [146]. SELENOM, a selenoprotein displaying antioxidant functions, was demonstrated regulate the cytosolic Ca^{2+} levels in the present study. Some of our preliminary data has also shown that SELENOM is co-expressed with IP3R1 and RyR3 in the wild-type mouse brain and the protein levels of IP3R1 and RyR3 are significantly reduced in the *Selenom*^{-/-} mouse brain. All these findings indicate that SELENOM may affect the redox environment and in turn, affect the calcium signaling via different calcium channels, IP3R and/or RyRs.

In conclusion, our findings provide new information in regard to the potential role of SELENOM in hypothalamic leptin signaling, ER stress, and Ca^{2+} signaling. We demonstrated that SELENOM deficiency impairs hypothalamic leptin signaling both *in vivo* and *in vitro*, indicating that SELENOM is a positive regulator of leptin signaling. Moreover, our data also revealed that SELENOM abrogates the induction of ER stress and Ca^{2+} store release in response to Tg.

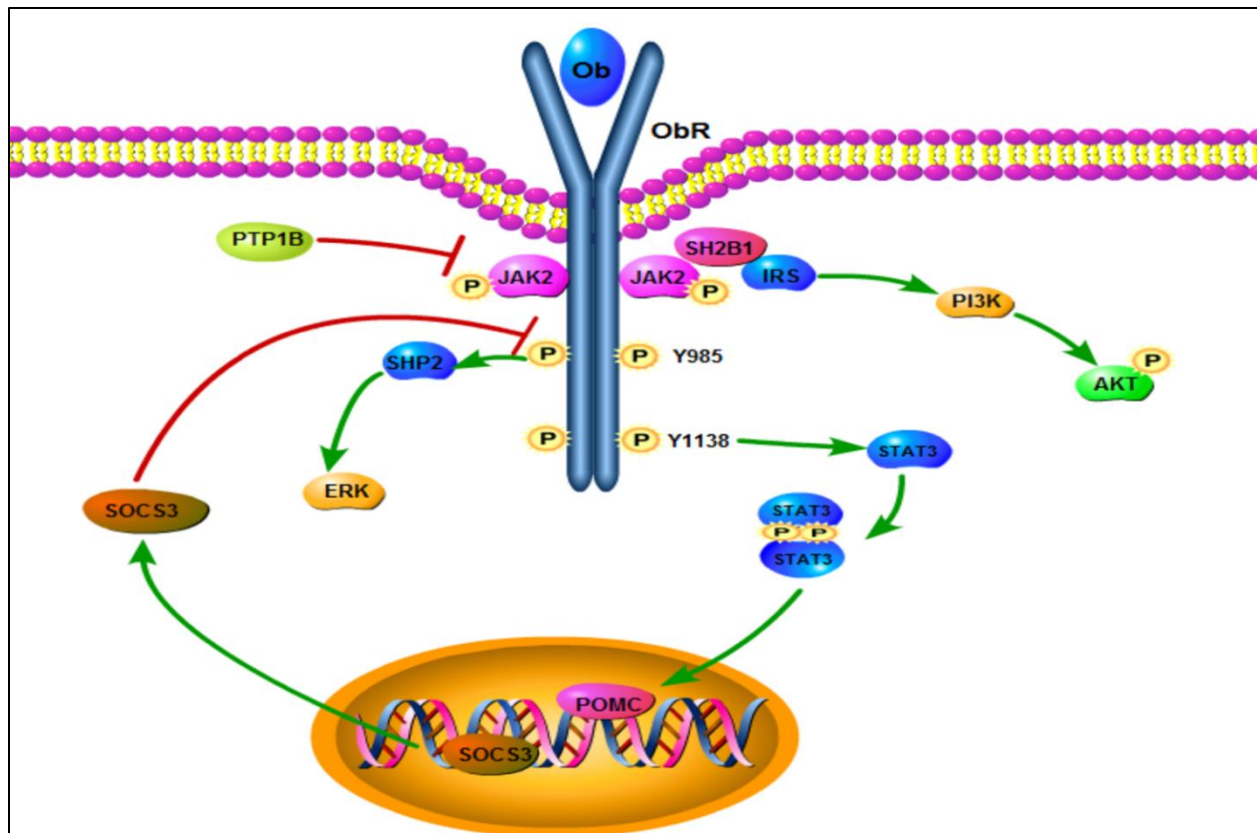
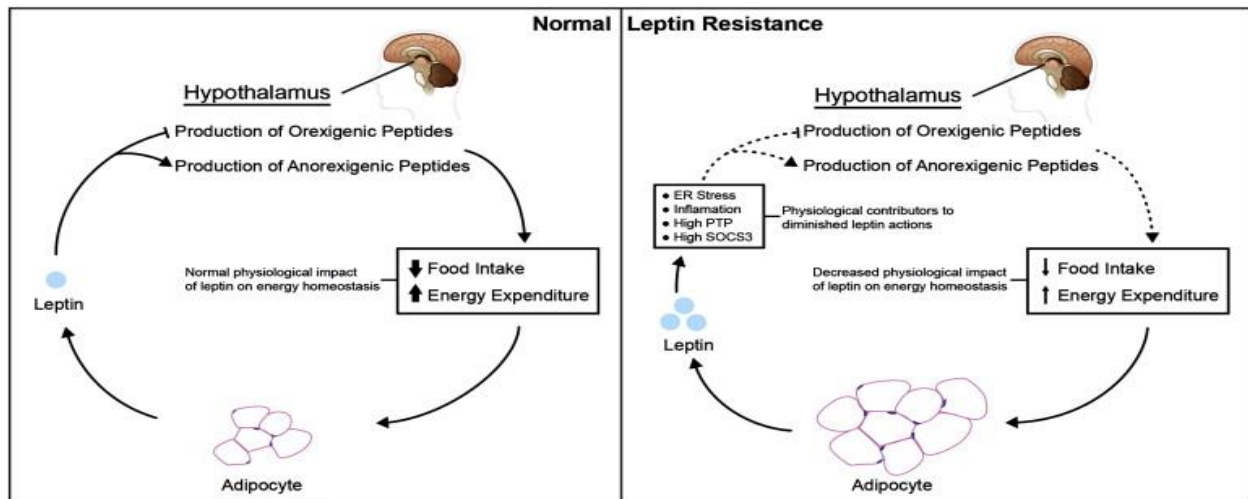


Figure 2-1. Summary of leptin signaling pathways. The binding between leptin (Ob) and leptin receptor (ObR) stimulates ObR dimerization and autophosphorylation of JAK2. In turn, JAK2 phosphorylates two key tyrosine residues on the ObR, Ty985 and Tyr1138, and then activate ERK and Stat3, respectively. In parallel, JAK2 can also activate IRS, PI3K, and AKT pathway. When phosphorylated, Stat3 forms a dimer and translocates into the nucleus, where it regulates the transcription of target genes, including POMC and SOCS3. SOCS3 and PTP1B are negative regulators of leptin signaling.



Adapted from St-Pierre et al., 2012

Figure 2-2. Summary of normal and leptin resistance physiology. In normal conditions, leptin derived from adipose tissue, plays a critical role in the regulation of food intake and energy expenditure. It increases the expression of anorexigenic peptides and inhibits the expression of the orexigenic peptides. On the contrary, leptin resistance in the hypothalamus is a major reason for the failure of leptin to reduce feeding behavior and stimulate energy metabolism.

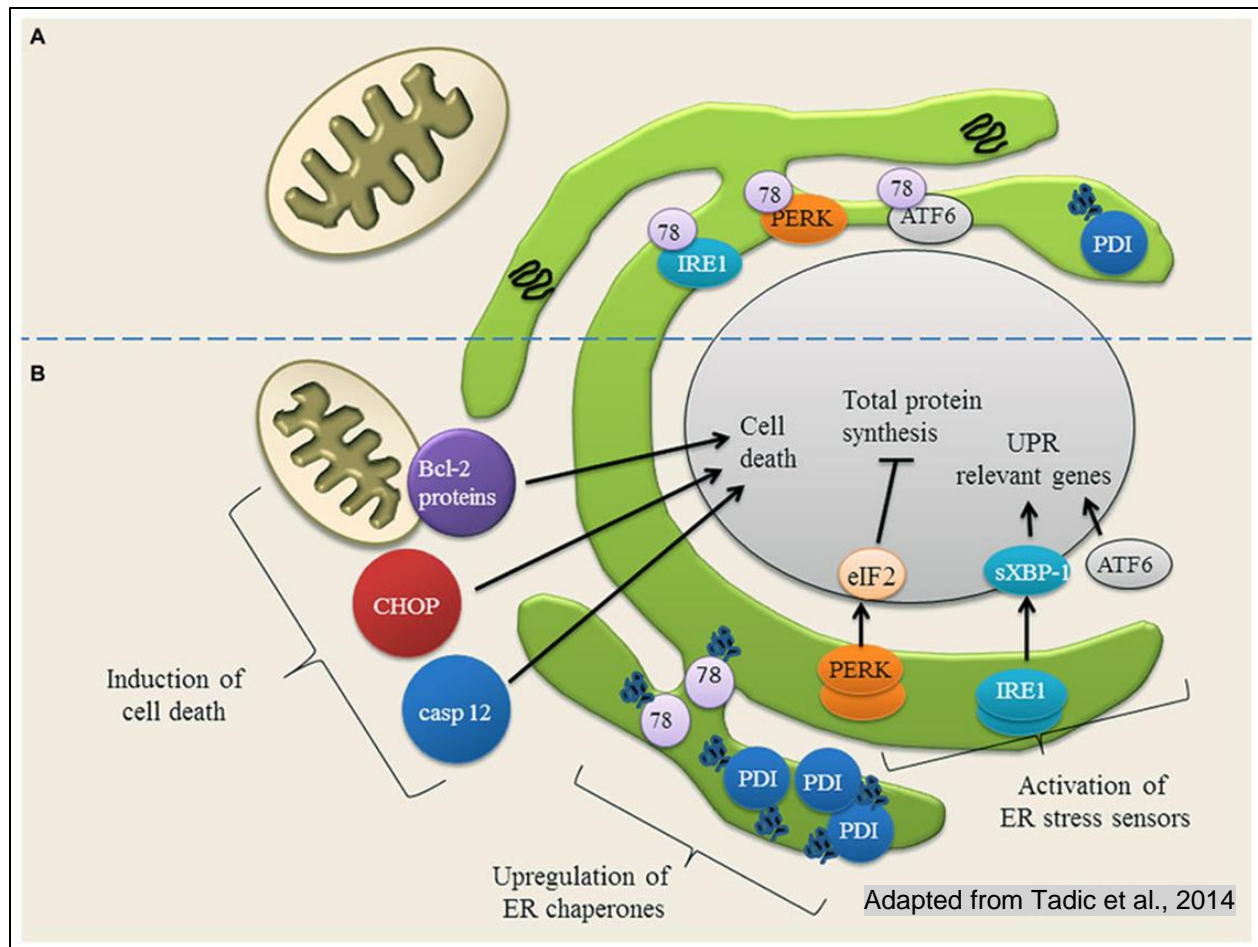


Figure 2-3. Summary of normal and ER stress conditions. ER stress can activate a variety of cellular processes via signaling pathways termed the unfolded protein response (UPR) in order to restore ER functions. In an unstressed state, these three proteins are inactive via association with glucose-regulated protein 78 (GRP78), the most abundant chaperone in the ER. In response to accumulation of unfolded proteins, GPR78 dissociates from these three ER stress sensors and translocates to the ER lumen for protein folding. This dissociation changes the conformation of PERK, IRE1 α , and ATF6, leading to their activation

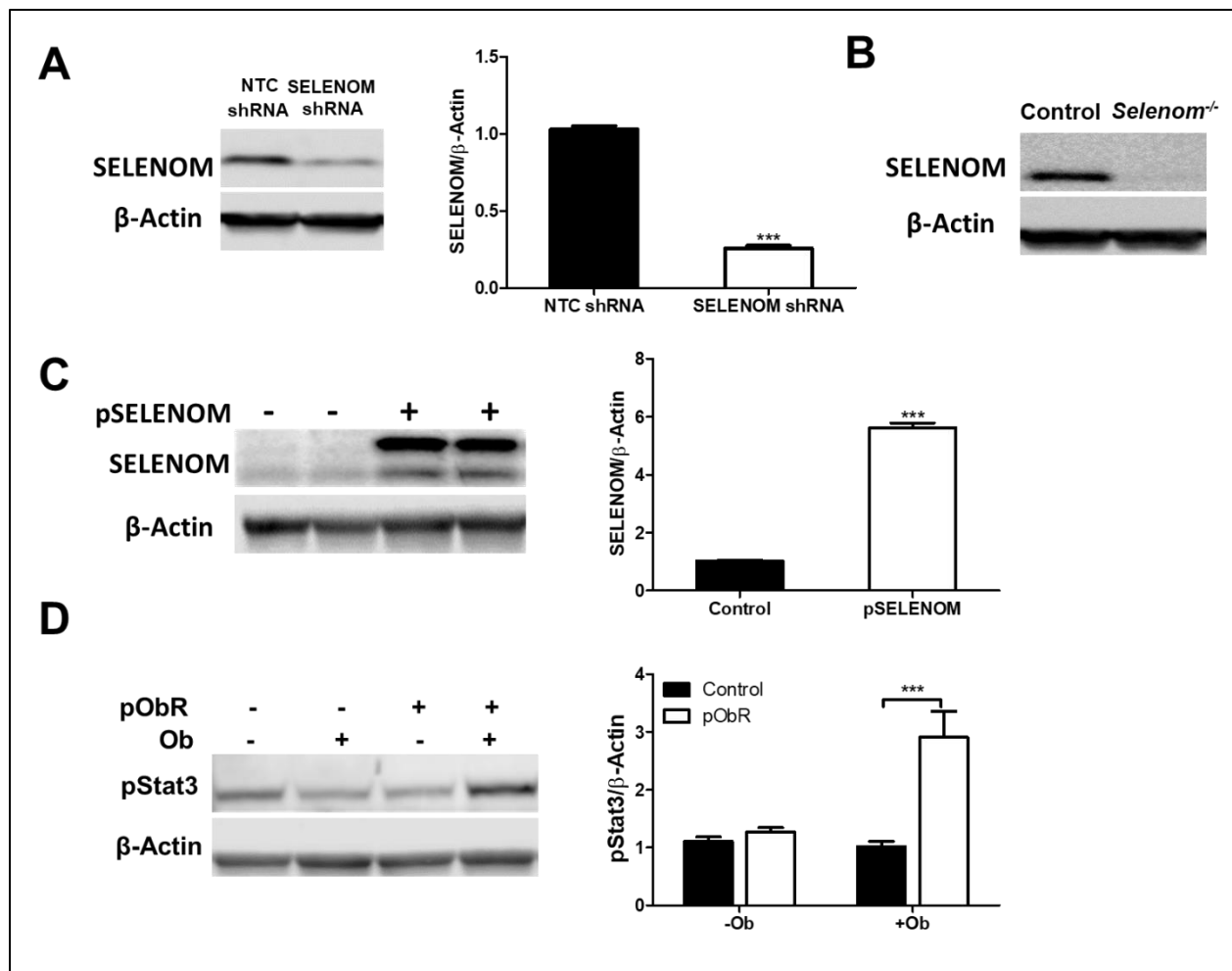


Figure 2-4. Manipulation of SELENOM expression in mHypoE-44 cells. **A**, Western blot analysis of SELENOM expression with shRNA-mediated knockdown. **B**, Western blot analysis of SELENOM expression with CRISPR/Cas9-mediated knockout. **C**, Western blot analysis of SELENOM expression in SELENOM-overexpressing (+pSELENOM) cells. **D**, Western blot analysis of leptin (Ob) signaling with Ob overexpressing (pObR) cells. Results shown represent the means \pm SEM (unpaired Student's t-tests or two-way ANOVA with Bonferroni post-tests) for at least three independent experiments. *** $p < 0.0001$, compared to control group.

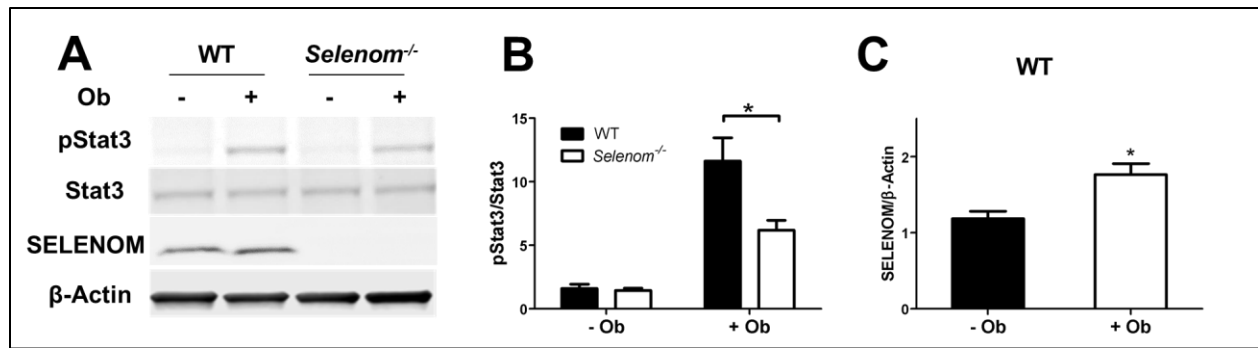


Figure 2-5. SELENOM is a leptin-responsive gene that promotes hypothalamic leptin signaling *in vivo*. **A**, Western blot analysis of leptin-induced signaling in hypothalamic tissue derived from 10 week old male wild-type and *Selenom*^{-/-} mice of comparable body weight. Animals were fasted overnight, challenged with an intraperitoneal injection of leptin (Ob) (1 µg leptin / gram body weight), and sacrificed 60 min thereafter (n = 3-4). **B**, Leptin-induced Stat3 phosphorylation was significantly diminished in *Selenom*^{-/-} samples. **C**, Leptin treatment significantly elevated SELENOM protein levels in wild-type samples. Results shown represent the means ± SEM (two-way ANOVA with Bonferroni post-tests) for at least three independent experiments. **p*<0.05, compared to control group.

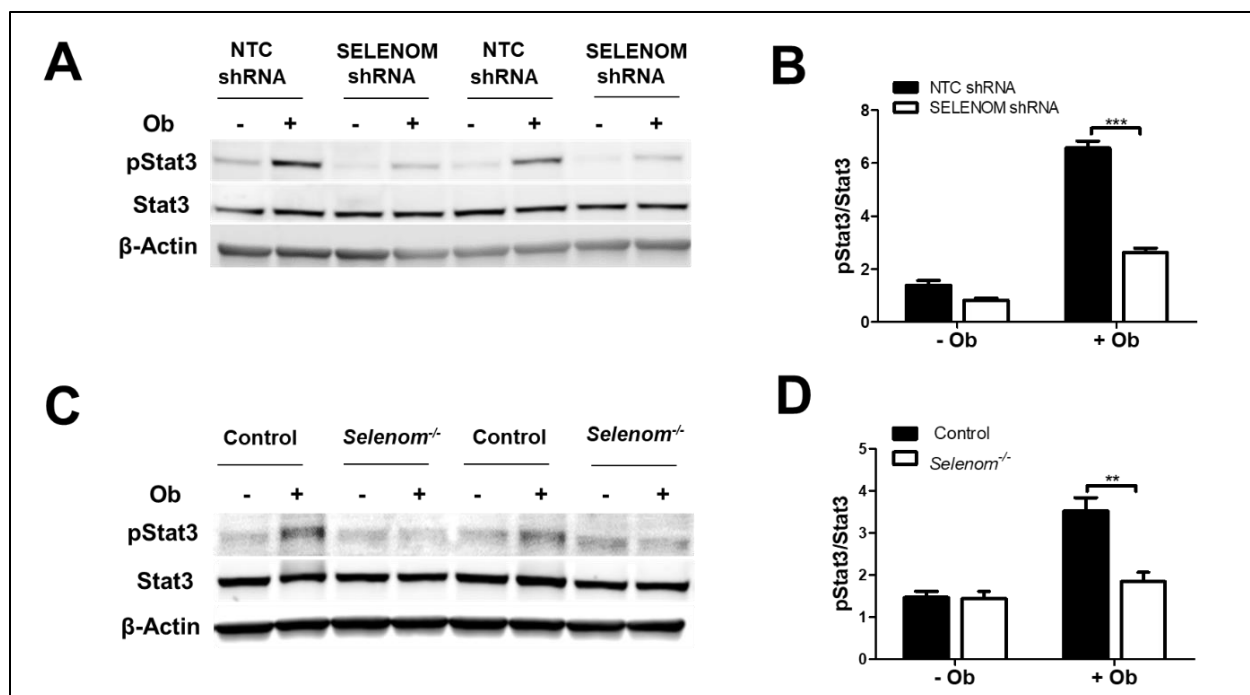


Figure 2-6. SELENOM promotes leptin signaling in mHypoE-44 cells. **A, C,** Western blot analysis of leptin-induced signaling in mHypoE-44 cells. Cells were serum-starved for 4 hours and then challenged with 100 nM leptin for 45 min. **B, D,** Levels of leptin-induced Stat3 phosphorylation was significantly reduced in samples with both shRNA-mediated knockdown (**B**) and CRISPR/Cas9-mediated knockout (**D**) of SELENOM as compared to the NTC shRNA control and normal control cells, respectively. Results shown represent the means \pm SEM (two-way ANOVA with Bonferroni post-tests) for at least three independent experiments. ** $p < 0.01$, *** $p < 0.001$ compared to control group.

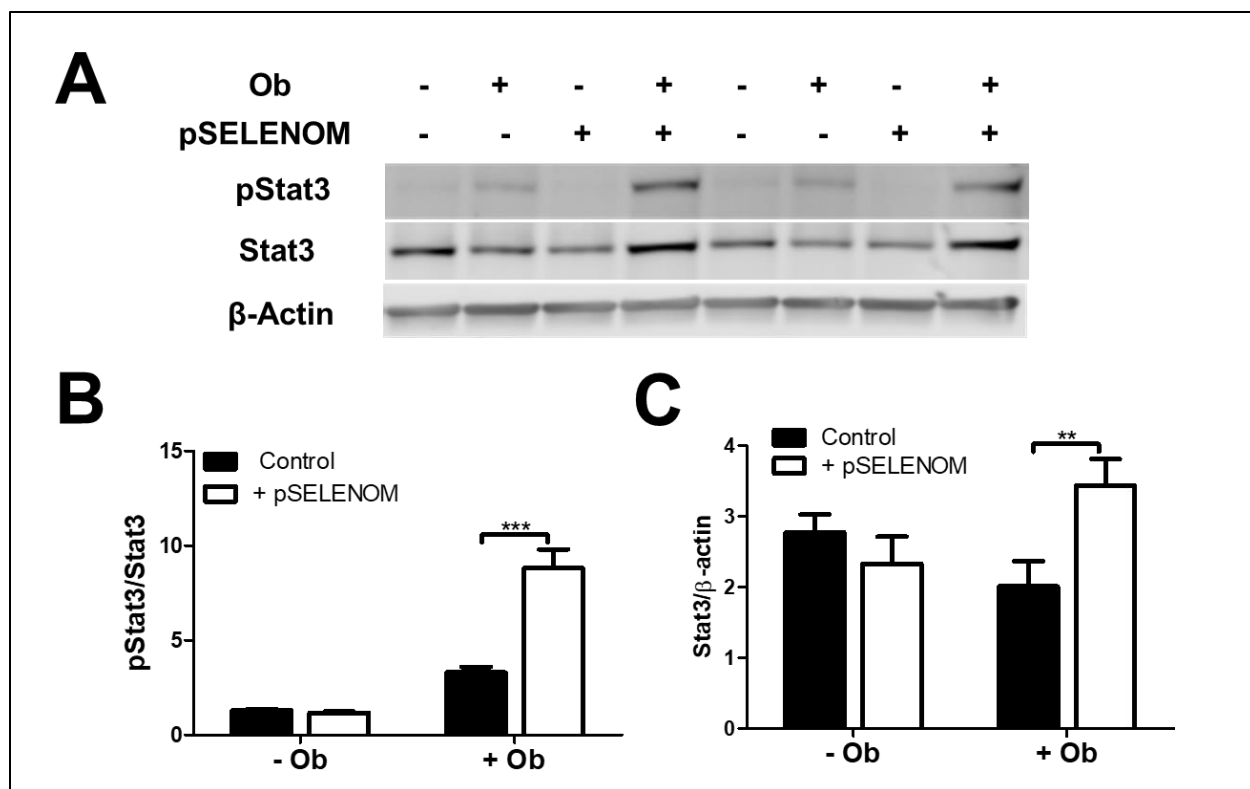


Figure 2-7. SELENOM overexpression promotes leptin signaling in mHypoE-44 cells. **A**, Treatment with SELENOM overexpression plasmid promotes leptin-stimulated increase in pStat3. **B**, Quantification of Stat3 phosphorylation normalized to total Stat3 levels. **C**, Quantification of Stat3 levels normalized to β -Actin levels. Results shown represent the means \pm SEM (two-way ANOVA with Bonferroni post-tests) for at least three independent experiments. ** $p < 0.01$, *** $p < 0.001$ compared to control groups.

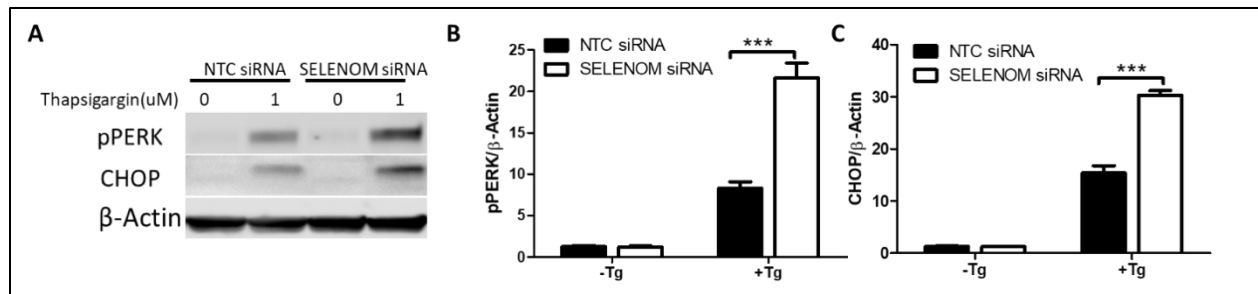


Figure 2-8. Treatment with SELENOM siRNA augments Tg-induced ER stress response A, Treatment with SELENOM siRNA augments Tg-induced increase in p-PERK and CHOP. **B,** Quantification of PERK phosphorylation normalized to β-Actin levels. **C,** Quantification of CHOP levels normalized to β-Actin levels. Results shown represent the means ± SEM (two-way ANOVA with Bonferroni post-tests) for at least three independent experiments. ***p<0.001 compared to control groups.

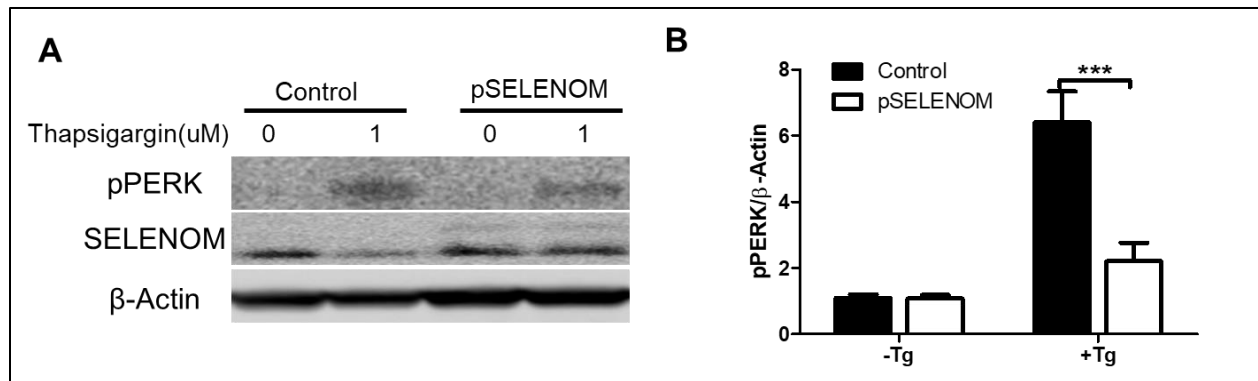


Figure 2-9. Overexpression of SELENOM reduces Tg-induced ER stress response. A, Overexpression of SELENOM reduces Tg-induced ER stress response in mHypoE-44 neurons. **B,** Quantification of PERK phosphorylation normalized to β -Actin levels. Results shown represent the means \pm SEM (two-way ANOVA with Bonferroni post-tests) for at least three independent experiments. ***p<0.001, compared to control groups.

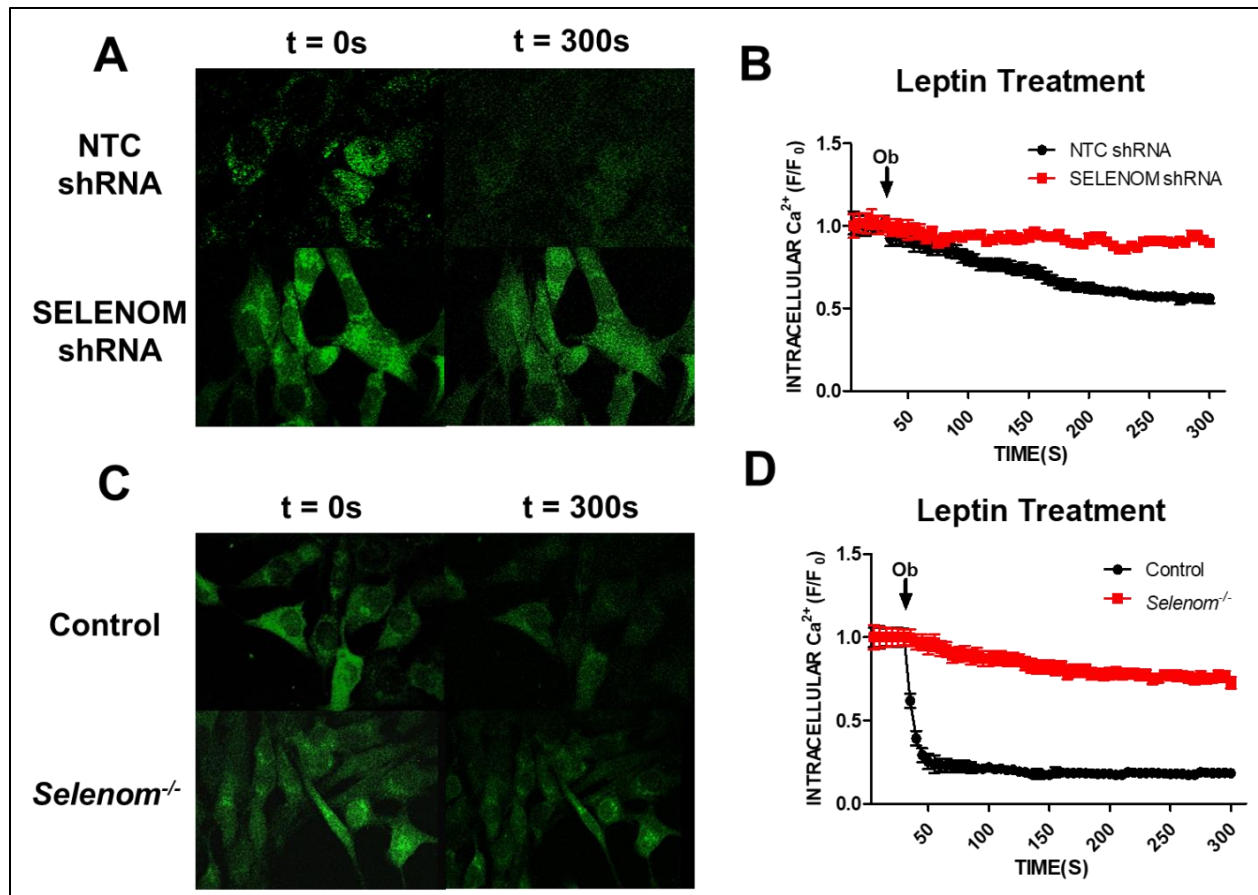


Figure 2-10. SELENOM deficiency impedes leptin-induced reduction of cytosolic Ca^{2+} levels in mHypoE-44 cells. **A, C,** Confocal images showing cytosolic Ca^{2+} levels before ($t = 0\text{s}$) and after treatment with leptin ($t = 300\text{s}$). **B, D,** Both shRNA-mediated knockdown (**B**) and CRISPR/Cas9-mediated knockout (**D**) of SELENOM impeded the leptin-induced reduction of cytosolic Ca^{2+} observed in control samples. Results shown represent the means \pm SEM for at least three independent experiments.

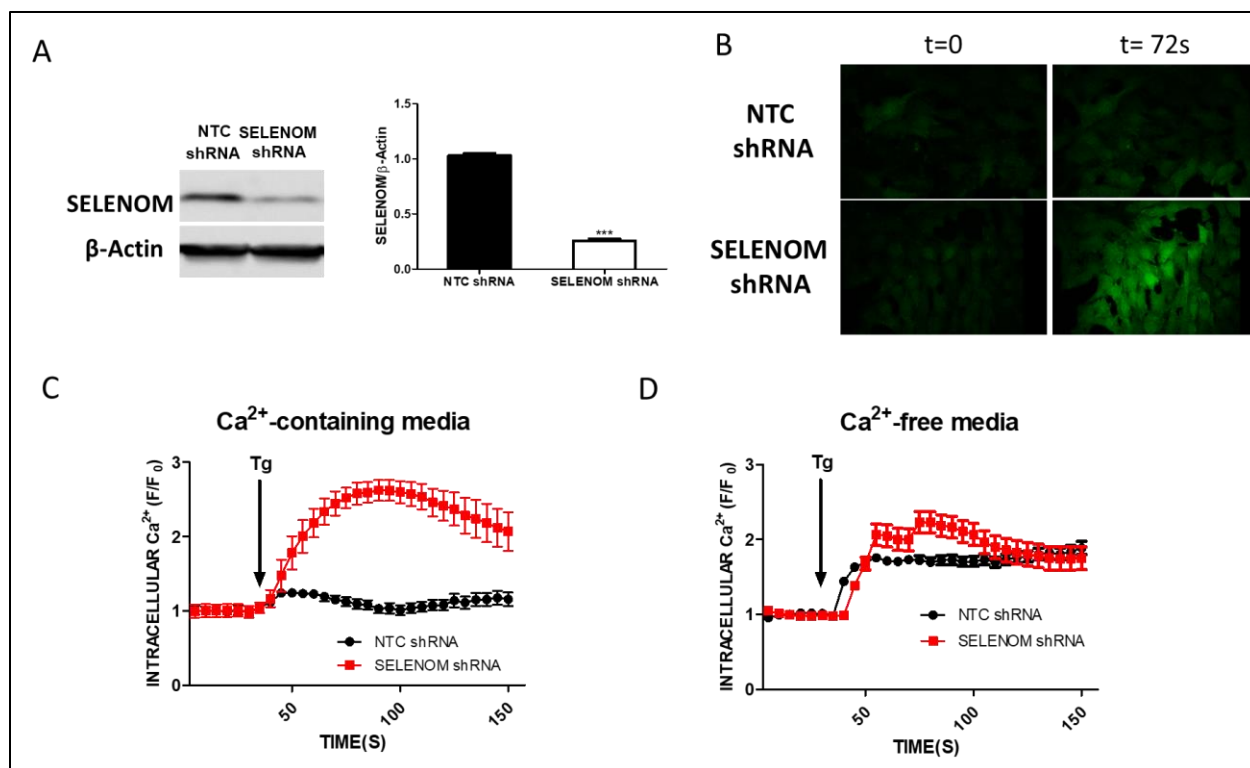


Figure 2-11. SELENOM knockdown in mHypoE-44 cells alters Ca^{2+} release from intracellular stores in response to Tg. **A**, SELENOM level is reduced after 48 hr DOX treatment in cells stably transfected with SELENOM shRNA. **B**, Ca^{2+} levels at baseline ($t = 0s$) and peak responses ($t = 72s$) to Tg in each group. **C**, SELENOM knockdown increases cytosolic Ca^{2+} levels to Tg (2 μ M). **D**, Cytosolic Ca^{2+} responses to Tg when cells were incubated in PBS without Ca^{2+} . Results shown represent the means \pm SEM for at least three independent experiments.

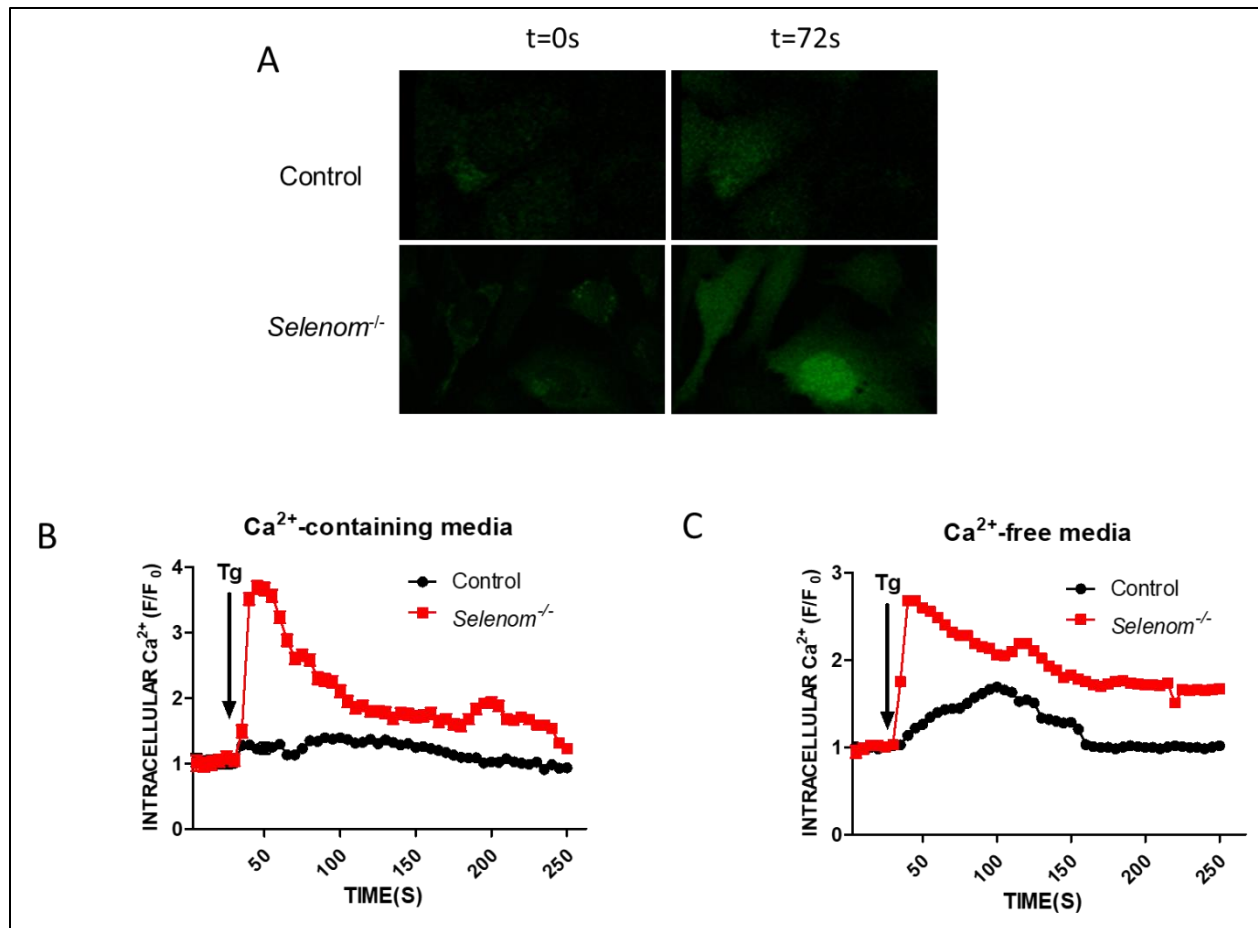


Figure 2-12. CRISPR/Cas9-mediated *Selenom*^{-/-} in mHypoE-44 cells alters Ca²⁺ release from intracellular stores in response to Tg. A, Ca²⁺ levels at baseline (t = 0s) and peak responses (t = 72s) to Tg in each group. **B,** *Selenom*^{-/-} increases cytosolic Ca²⁺ levels to Tg (2 μ M) **C,** Cytosolic Ca²⁺ responses to Tg when cells were incubated in PBS without Ca²⁺. Results shown represent the means \pm SEM for at least three independent experiments.

CHAPTER 3. SUBCELLULAR LOCATION AND INTERACTION PARTNERS OF SELENOM

Abstract

SELENOM is an ER-resident thiol-disulfide oxidoreductase implicated in the Ca^{2+} homeostasis, ER stress, and energy metabolism. ER is well known as the major storage site for Ca^{2+} by which ER can interact and share information with the cytosol and other organelles, such the mitochondria. Ca^{2+} flux from the ER to the mitochondria plays an essential role in regulating both ER and mitochondrial functions. Therefore, ER-mitochondria connection sites, named mitochondria associated ER membranes (MAMs), are essential for many biological processes that occur in these two organelles. In the present studies, we performed subcellular fractionation using wild-type mouse brains and found that SELENOM was not only detected in ER, but also localized in MAMs and the mitochondria. We then conducted a screening of SELENOM-binding partners using co-immunoprecipitation and LC-MS/MS. Results determined two interaction partners of SELENOM, microtubule associated protein 6 (MAP6) and phosphate cytidylyltransferase 1, choline, alpha (PCYT1A), which are indicated in Ca^{2+} signaling and phosphatidylcholine biosynthesis.

Introduction

The ER is a central organelle responsible for lipid biosynthesis, protein folding, and post translational modifications. Moreover, the ER is the major storage site for Ca^{2+} , by which it can export signal information to the cytosol and other organelles, the mitochondria in particular [147, 148]. Ca^{2+} flux from the ER to the mitochondria plays an essential role in regulating mitochondrial functions, including energy metabolism, Ca^{2+} homeostasis, and apoptotic signaling. Elevated levels of Ca^{2+} within the mitochondria promotes the Krebs cycle, adenine nucleotide transporters, and pyruvate dehydrogenase, leading to increased ATP production [149-152]. The accumulation of misfolded protein leads to increased ER-mitochondria contact and elevated Ca^{2+} efflux to the mitochondria, thereby stimulating energy production [153]. However, high levels of Ca^{2+} can also induce modifications in mitochondria membrane potential

and permeability, leading to apoptosis via cytochrome C [138]. Dysregulation of the Ca^{2+} flux during this process has been involved in many disorders, including metabolic diseases, cancer, and neurodegeneration.

Therefore, Ca^{2+} flux modulated by ER-mitochondria connection sites is essential for biological processes occurring in these two organelles. The physical interaction sites between the ER and mitochondria, first discovered in 1950 by electron microscopy in rat tissues, are termed as mitochondria associated ER membranes (MAMs) [148]. These highly dynamic structures regulate the bidirectional exchange of lipids, Ca^{2+} , and other metabolites. Moreover, MAMs are implicated in mitochondria bioenergetics and morphology, therefore coordinating the homeostasis between ER and mitochondria.

Herein, we set out to assess SELENOM subcellular localization and speculate that SELENOM may also localize in MAMs which are involved in Ca^{2+} regulation. Moreover, SELENOM may have some potential interaction partners related to Ca^{2+} homeostasis and MAMs regulation. In these present studies, we found that SELENOM was not only detected in ER, but also localized in MAMs and mitochondria. Interestingly, we found two interaction partners of SELENOM, MAP6 and PCYT1A, which are involved in Ca^{2+} signaling and phosphatidylcholine biosynthesis.

Methods and Materials

Chemicals and antibodies

Primary antibodies used in these experiments are rabbit anti-COX-IV (1:1000; Cell signaling, 4850), mouse anti-VDAC1 (1:500; Santa Cruz, sc-390996), rabbit anti-IP3R (1:500; Santa Cruz, sc-377518), mouse anti-SERCA2 (1:500; Santa Cruz, sc-376235), mouse anti-BRCA1 (1:500, Santa Cruz, sc-6954), mouse anti-STOP (1:500, Santa Cruz, sc-53513), mouse anti- PCYT1A (1:500, Santa Cruz, sc-376107), rabbit anti-PDI (1:1000, cell signaling,2446), mouse anti-FACL-4 (1:500, Santa Cruz, sc-365230), and mouse anti- β -Actin (1:5000; Cell Signaling, 3700). Species specific secondary antibodies were purchased from LI-COR.

Animals

Male wild-type and *Selenom*^{-/-} mice based on C57BL/6N background were born, bred, and raised in the University of Hawai'i Vivarium (N≥5 for each group). Mice were sacrificed with CO₂ at week 10 and mouse whole brains were snap-frozen for future analysis. The method of hypothalamic dissection was described in Chapter 2.

Cell culture

The hypothalamic cell line mHypoE44 (Cellution) and CRISPR/Cas9 stable knockout cell line (Described in Chapter II) were maintained in DMEM (Sigma) with 10% FBS (Invitrogen) and 1% penicillin in a humidified incubator with 5% CO₂ at 37°C.

Subcellular fractionation

Mouse brains were homogenized and centrifuged twice at 800 x g to remove unbroken cells and nuclei. Supernatants were then subjected to centrifugation three times at 10,000 x g to remove the plasma membrane, lysosomes, microsomes, and cytosol. The collected pellet containing the crude brain mitochondria was resuspended and transferred to an ultracentrifuge tube containing Percoll and mitochondria resuspending buffer. We centrifuged the samples at 95,000 x g to separate pure mitochondria from mitochondria-associated membranes (MAMs). A dense band containing the purified mitochondria fraction localizes at the bottom with a white band containing the MAMs fraction above it. These bands were further washed and collected to get pure mitochondria and MAMs. Fractions from the experiments above were subjected to Western Blot to verify the purity (Figure 3-1).

Co-immunoprecipitation and mass spectrometry

Brain samples were lysed in CellLytic MT (Sigma) with protease/phosphatase inhibitors (Cell signaling) and subject to a centrifugation at 14,000 x g for 15 minutes to remove cellular debris. Whole cell lysates (200 µg) were pre-cleared by incubating with Magnetic Protein G-coupled Dynabeads (Thermo Fisher Scientific Life Science) for 10 minutes. Meanwhile, magnetic Protein G-coupled Dynabeads (50 µl) were incubated with 3µg SELENOM Abs for 10 minutes, as recommended by the manufacturer. After crosslinking the antibody to Dynabeads using BS³, pre-cleared lysates were

incubated with Dynabeads (50 μ l) bound to the SELENOM antibody overnight at 4°C. Unbound protein samples were collected and the protein complex were eluted from beads the following day. Eluates were separated via SDS/PAGE and the polyacrylamide gels were stained with SilverXpress™ silver staining kit (Thermo Fisher Scientific Life Science) according to the manufacturer's instruction. Three bands with visible difference between two groups were excised and sent out for LC-MS/MS analyses by Applied Biomics (Hayward, CA, USA).

Western Blot

Described in Chapter II.

Statistical analysis

Described in Chapter II.

Results

Identification of SELENOM subcellular localization. We speculated that SELENOM may also reside at the site of physical communication between ER and mitochondria, MAMs. To examine this notion, we isolated ER, MAMs, crude mitochondria, and pure mitochondria fractions from wild-type mouse brains following a well-established subcellular fractionation protocol described in methods section. The subcellular fractions and whole cell lysates were prepared and then subjected to Western blot analysis, probing for specific organelle markers, including Long-chain fatty-acid CoA synthases (FACL-4) as a MAMs marker, cytochrome c oxidase subunit 4 (COX4) as a mitochondria marker, Voltage-dependent anion channel 1 (VDAC1) as a mitochondria and MAMs marker, and protein disulfide isomerase (PDI) as an ER marker [154-157]. VDAC1, as a mitochondrial and MAMs marker, should be expressed in the crude mitochondria fraction and extremely enriched in the pure mitochondria fraction. It could also be present in the MAMs fraction. The ER marker, PDI, localizes in the ER and plays essential role in protein folding. Western blot analysis shows that the MAMs fraction is enriched for FACL-4 and pure mitochondria fraction is enriched in VADC1, indicating the high purity of the MAMs and pure mitochondria

(Fig. 3-1). Our data also verified the purity of the crude mitochondria and ER fractions. SELENOM expression was detected in the ER, MAMs, and mitochondria, suggesting a new subcellular map of SELENOM. These results are also consistent with the notion that SELENOM localizes in the MAMs and may potentially contribute to the transportation of Ca^{2+} and other metabolites between the ER and mitochondria.

Identification of SELENOM interacting partners. Some studies have revealed interaction partners of SELENOM, such as Galectin-1, Notch2nl, Nenf proteins, and Mtus proteins [158-160]. However, there have been no further studies on the functions and underlying mechanisms of SELENOM and its interactions partners. Herein, we anticipate that SELENOM may interact with proteins that contribute to leptin signaling, Ca^{2+} regulation, and energy metabolism. A screening of SELENOM-binding partners was conducted using protein derived from 10-week-old wild-type mouse brain with corresponding protein from *Selenom*^{-/-} mouse brain as a negative control. Lysates from both samples were incubated with anti-SELENOM-coated beads, which co-immunoprecipitate (co-IP) SELENOM and its binding partners. Target protein complexes were then eluted and subjected to SDS-PAGE, followed by silver staining (Fig. 3-2A). Three prominent bands were observed and hence excised from both wild-type and *Selenom*^{-/-} samples at molecular weights corresponding to 40kDa, 70kDa, and 140 kDa for further analysis. These gel slices samples were then digested and analyzed by LC-MS/MS for potential binding partners. This analysis led to the identification 15 SELENOM-interaction partners that only appeared in the wild-type samples (Table 2). To further verify the interactions, we first chose the proteins of which molecular weight are close to 40kDa, 70kDa, and 140 kDa and performed a literature search of their known function. We found that several proteins are largely involved in the energy metabolism, including PCYT1A, MAP6, and Brca1. The same co-IP experiments using anti-SELENOM-coated beads were conducted and followed by Western blot analysis, probing for those potential interacting proteins of SELENOM. Results confirmed two candidates of particular interest, MAP6 and PCYT1A. We also conducted co-IP experiments using anti-MAP6-coated beads to further confirm the interaction between SELENOM and MAP6 by Western blot

analysis (Fig. 3-2B, C). Interestingly, MAP6 was also detected when pulling down PCYT1A, suggesting a potential protein complex formed by these three proteins (data not shown).

Discussion

Ca²⁺ released from the ER to the mitochondria plays a critical role in several mitochondrial functions and is a major determinant of energy metabolism and cellular fate. As the main storage compartment for Ca²⁺, the ER maintains a specific environment containing chaperones and folding enzymes for protein folding and maturation. All these biological processes require a high concentration of Ca²⁺ in the ER which is maintained by the ER Ca²⁺ uptake system, SR/ER Ca²⁺ transport ATPase b (SERCA2b). This store will release Ca²⁺ through two gated Ca²⁺ release channels, inositol 1, 4, 5-trisphosphate receptors (IP3Rs) and the ryanodine receptors (RyRs) in response to various stimulation.

The ER forms abundant membrane contact sites with the plasma membrane and the other organelles, including mitochondria, endosomes, Golgi, lysosomes, lipid droplets, and peroxisomes [161]. Electron microscopy and fluorescence microscopy of the MAMs in cells and yeast reveal a cleft of 10-50 nm between the ER and mitochondria [162]. However, the physical structures of MAMs are dynamic and highly regulated by different status of cells. In addition, the ER-mitochondria contacts become closer in the early phases of ER stress [163]. Csordas et al. enhanced the ER-mitochondria association using a synthetic linker protein and found that bringing the ER closer to the mitochondria significantly increases mitochondria Ca²⁺ uptake [164]. All these findings delineate the tight relationships of MAMs and Ca²⁺ regulation. However, the mechanisms by which MAMs undergoes this plasticity is still elusive and the impact on the mitochondrial Ca²⁺ is highly variable.

ER-resident selenoproteins include SELENOF, SELENOK, SELENOM, SELENON, SELENOS, SELENOT, and the type-2 iodothyronine deiodinase. Among these selenoproteins, SELENOK, SELENOM, and SELENON have been indicated in regulating Ca²⁺ signaling [95, 100, 101, 165]. SELENOK was reported as an ER-membrane protein that promotes Ca²⁺ influx during activation of immune cells, and SELENOK deficiency causes impaired Ca²⁺ flux due to a defect in palmitoylation of

inositol-1, 4, 5-triphosphate receptor (IP3R), a Ca^{2+} channel protein. SELENOM was implicated in ER redox balance and Ca^{2+} homeostasis via SERCA2 pump [101]. The function of a protein is closely correlated with its subcellular location. Herein, we performed subcellular fractionation to verify the compartmentalization of SELENOM. We showed that, in addition to localizing to the ER (and Golgi), SELENOM can also be detected in MAMs and mitochondria, suggestive of a role in regulating Ca^{2+} flux between the ER and mitochondria. Although the precise mechanism has yet to be established, it is highly possible that SELENOM regulates Ca^{2+} flux from ER to mitochondria by affecting specific ER Ca^{2+} channels via a redox mechanism.

Structure studies of SELENOM suggest that the flexible C-terminal region is a potential binding site of SELENOM for protein substrates [78]. Moreover, a 3D structure model of SELENOM reveals three well conserved residues (F59, L82, and L84) that act as putative hub nodes for retaining pivotal protein-protein interactions [79]. In a previous study, SELENOM with a modified structure was used to screen for the potential interaction partners by the yeast two-hybrid system. The results suggested an interaction between SELENOM and Galectin-1, a protein which prevents neurodegeneration [159]. However, in our co-IP experiments using mouse brain samples, we could not reproduce the same results. This may be due to environmental differences between the yeast two-hybrid system and endogenous mouse brain tissue. Our LC-MS/MS analysis and further co-IP experiments determined two binding partners of SELENOM, MAP6 and PCYT1A. MAP6 encodes microtubule-associated protein 6 and is related to microtubule stabilization under different conditions, including low temperature and treatment of depolymerizing reagents [166, 167]. *MAP6*^{-/-} mice display severe behavioral disorders that are associated with synaptic plasticity defects [168]. The functions of MAP6 are largely regulated via its phosphorylation by Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), which is regulated by Ca^{2+} /calmodulin complex and is necessary for Ca^{2+} homeostasis [169, 170]. It has been reported that Ca^{2+} /calmodulin competes with the microtubule for binding sites of MAP6 during synaptic activation [171]. A more recent report found that MAP6 interacts directly with both T-Complex-Associated-Testis-Expressed 1 (Tctex1) and the C-terminus of Cav2.2/N-type Ca^{2+} channels, indicating a specific role of MAP6 to modulate Ca^{2+} signaling in neurons [172]. This study also suggests that MAP6 is largely related to Ca^{2+} signaling in neurons.

PCYT1A, phosphocholine cytidyltransferase, is an essential rate-limiting and regulatory enzyme in phosphatidylcholine (PC) synthesis. PC is the most abundant phospholipid and maintains the structure and functionality of all cellular membranes. PCYT1A, largely resident in the nucleus, becomes more active form on translocating to the ER membrane for PC biosynthesis [173]. Interestingly, PC biosynthesis and PCYT1A have been largely correlated with ER stress pathways. Inhibition of PC biosynthesis using a thermo-sensitive mutation in PCYT1A at non-permissive temperature led to PC depletion, and in turn induced the expression of ER-stress-related protein CHOP, indicating that PC depletion by inactivation of PCYT1A may induce an ER stress response [174]. Upon the activation of UPR, XBP-1 is spliced to the active form XBP-1s which acts as a transcription factor and regulates the transcription of genes involved in ER membrane biosynthesis, protein transportation [175]. Sriburi et.al reported that XBP-1s-induced ER biogenesis in fibroblasts enhanced PC biosynthesis and increased activities of PCYT1A and CPT [175]. Further experiments revealed that the effect of XBP-1s in the CDP-choline pathway is primarily based on its regulation of PCYT1A activity [83]. PC is also the major phospholipid of mitochondrial membranes and needs to be imported from other organelles, mainly from ER [176]. During transport, PC can be detected at contact sites between ER and mitochondria [176, 177], suggesting MAMs are a key site involved in lipid translocation to mitochondria [178]. SELENOM, localizing in MAM and interacting with PCYT1A, may potentially affect the synthesis and transport of PC from ER to mitochondria, and in turn, modulate the dynamic morphology and functions of mitochondria.

We found that SELENOM localizes to MAMs, which suggests that SELENOM may regulate Ca^{2+} signaling between ER and mitochondria. Moreover, we identified two interaction partners of SELENOM, MAP6 and PCYT1A, which are involved in Ca^{2+} signaling and phosphatidylcholine biosynthesis.

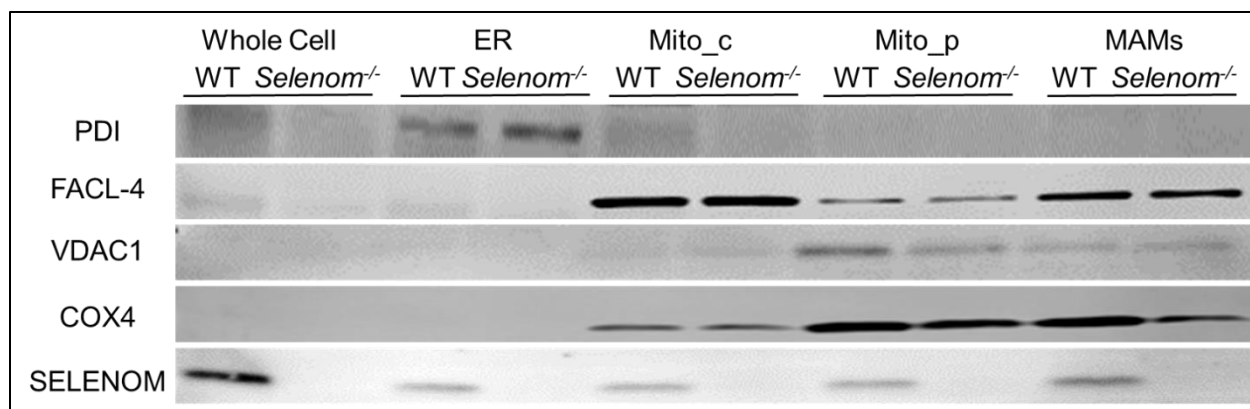


Figure 3-1. Subcellular localization of SELENOM in mouse brain. Western blot of subcellular fractions prepared from wild-type and *Selenom*^{-/-} brain. Proteins were detected with rabbit anti-PDI (ER marker), mouse anti-FACL-4 (MAMs marker), mouse anti-VDAC1 (mitochondria and MAMs marker), rabbit anti-COX 4 (mitochondria marker), and mouse anti-SELENOM antibodies. ER: endoplasmic reticulum; Mito_c: crude mitochondrial fraction; Mito_p: pure mitochondrial fraction; MAMs: mitochondria-associated membranes. Results shown represent for at least three independent experiments.

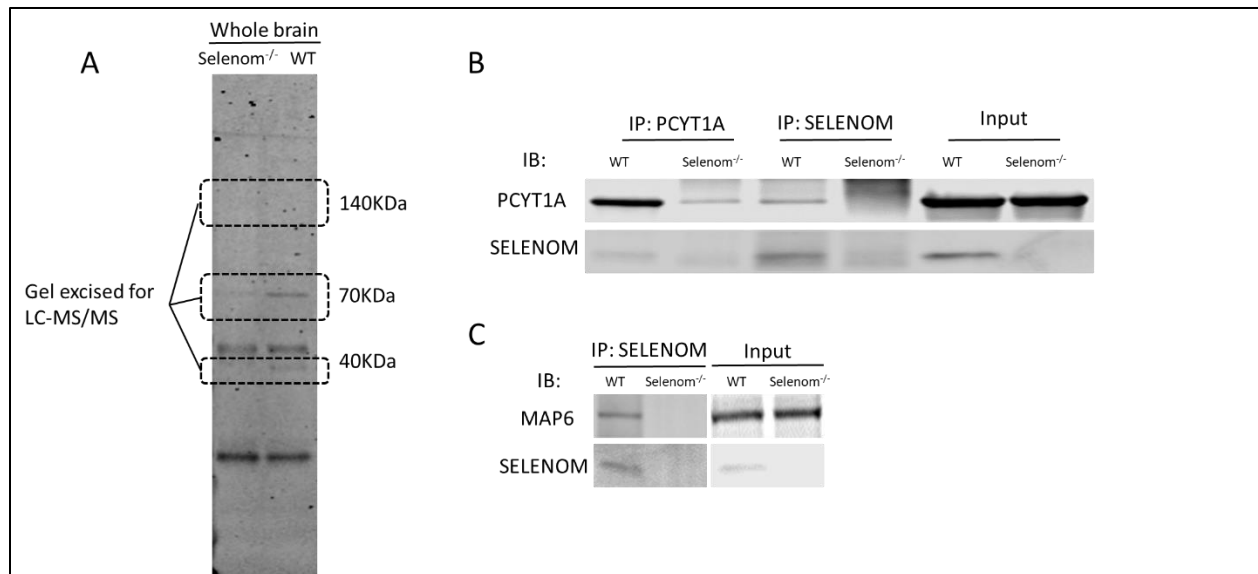


Figure 3-2. SELENOM interacts with MAP6 and PCYT1A in mouse brain. **A**, wild-type and *Selenom*^{-/-} brain lysates were incubated with anti-SELENOM coated magnetic beads overnight and eluted. Eluates were then separated by SDS-PAGE and stained by silver staining kit. Gels slices were excised for each lane around 40kDa, 70kDa, and 140kDa. **B**, Interactions between SELENOM and PCYT1A were investigated by Co-immunoprecipitation with wild-type and *Selenom*^{-/-} brain samples. **C**, Interactions between SELENOM and MAP6 were investigated by co-immunoprecipitation with wild-type and *Selenom*^{-/-} brain samples.

Table 2. Candidate interaction partners of SELENOM in wild-type samples

Gene Name	Accession No.	Protein Name	MW
Alms1	ALMS1_MOUSE	Alstrom syndrome protein 1 homolog OS=Mus musculus GN=Alms1 PE=1 SV=2	360
Brca1	BRCA1_MOUSE	Breast cancer type 1 susceptibility protein homolog OS=Mus musculus GN=Brca1 PE=1 SV=3	199
C1qb	C1QB_MOUSE	Complement C1q subcomponent subunit B OS=Mus musculus GN=C1qb PE=1 SV=2	27
capza3	CAZA3_MOUSE	F-actin-capping protein subunit alpha-3 OS=Mus musculus GN=Capza3 PE=2 SV=1	35
ccdc150	CC150_MOUSE	Coiled-coil domain-containing protein 150 OS=Mus musculus GN=Ccdc150 PE=2 SV=2	129
ccdc190	CC190_MOUSE	Coiled-coil domain-containing protein 190 OS=Mus musculus GN=Ccdc190 PE=2 SV=1	33
cfap74	CFA74_MOUSE	Cilia- and flagella-associated protein 74 OS=Mus musculus GN=Cfap74 PE=2 SV=3	179
col4a1	CO4A1_MOUSE	Collagen alpha-1(IV) chain OS=Mus musculus GN=Col4a1 PE=1 SV=4	161
Slc25a18	GHC2_MOUSE	Mitochondrial glutamate carrier 2 OS=Mus musculus GN=Slc25a18 PE=1 SV=4	34
Stop	MAP6_MOUSE	Microtubule-associated protein 6 OS=Mus musculus GN=Map6 PE=1 SV=2	96
Mycbpap	MYBPP_MOUSE	MYCBP-associated protein OS=Mus musculus GN=Mycbpap PE=1 SV=1	106
Nefn	NFH_MOUSE	Neurofilament heavy polypeptide OS=Mus musculus GN=Nefn PE=1 SV=3	117
Pcyt1a	PCY1A_MOUSE	Choline-phosphate cytidylyltransferase A OS=Mus musculus GN=Pcyt1a PE=1 SV=1	42
Rls1d1	RL1D1_MOUSE	Ribosomal L1 domain-containing protein 1 OS=Mus musculus GN=Rsl1d1 PE=1 SV=1	50
Wnt8a	WNT8A_MOUSE	Protein Wnt-8a OS=Mus musculus GN=Wnt8a PE=2 SV=2	40

*MW: molecular weight (kDa).

CHAPTER 4. SELENOM DELETION AFFECTS THE THIOREDOXIN SYSTEM

Abstract

As a member of TXN superfamily, SELENOM shares the similar structure (CXXU) with TXN at its active site and has been implicated in redox signaling and energy metabolism. In Chapter 2, we have presented evidence that SELENOM is involved in leptin signaling, induced ER stress, and Ca^{2+} signaling. However, SELENOM-related genes and genetic pathways have yet to be characterized. Herein, we performed microarray analysis using both hypothalamic tissue and mHypoE-44 cells to identify genes and signaling pathways mostly affected by SELENOM deficiency. Among the identified differentially expressed genes (DEGs), thioredoxin interacting protein (TXNIP) is of particular interest as its specific function is related to the TXN system. TXN and TXNRD activity were both found altered by SELENOM deficiency as well. Taken together, our findings demonstrate that hypothalamic TXN system is regulated by SELENOM and potentially contributes to hypothalamic leptin signaling.

Introduction

SELENOM is a thiol-disulfide oxidoreductase that is highly expressed in the brain. Specifically, our group has found that SELENOM is highly expressed in several discrete brain regions, including the arcuate and paraventricular nuclei of the hypothalamus and some other regions of brain. Among all these regions, paraventricular nucleus (PVN) and arcuate nucleus (ARC) of hypothalamus are of most interest due to their involvement in energy metabolism regulated by leptin. SELENOM is implicated in leptin signaling both *in vitro* and *in vivo*. *Selenom*^{-/-} mice exhibit significant weight gain with increased circulating leptin levels and impaired leptin sensitivity in the ARC of hypothalamus [81]. Aforementioned results in Chapter 2 have demonstrated that SELENOM is a leptin-responsive gene and can regulate leptin signaling, ER stress, and Ca^{2+} signaling.

Thus, to further investigate the genes and signaling pathways affected by SELENOM deletion, we conducted microarray analysis on both hypothalamic cell and tissue samples. Our analyses revealed that TXNIP was significantly downregulated in both hypothalamic tissue and mHypoE-44 cells. Moreover, TXN

activity was significantly decreased by SELENOM deficiency. Taken together, our studies verify that SELENOM regulates the TXN antioxidant activities, which may contribute to its function in hypothalamic leptin signaling.

Methods and Materials

Chemicals and antibodies

Primary antibodies used in these experiments are mouse anti-TXNIP (1:1000; NOVUS, NBP1-54578) and mouse anti- β -Actin (1:5000; Cell Signaling, 3700). Species specific secondary antibodies were purchased from LI-COR.

Animals

Described in Chapter 2.

CRISPR/Cas9-mediated *Selenom*^{-/-} in mHypoE-44 cells

Described in Chapter 2.

Microarray analysis

RNA was isolated from hypothalamic tissue and mHypoE-44 cells using the RNeasy lysis kit (Qiagen). Samples were then sent to the University of Hawai'i Cancer Center Genomics Core Facility for microarray analysis. RNA integrity was first validated on an Agilent 2100 Bioanalyzer. 100 ng of total RNA was then used for the GeneChip Whole-transcript assay followed by hybridization to Clariom S Mouse Arrays (Affymetrix, Santa Clara, CA, USA). Arrays were washed, stained, and scanned using a GeneChip Fluidics Station 450 and a GeneChip Scanner (Affymetrix). CEL files generated from Clariom S Mouse Arrays were analyzed by Transcriptome Analysis Console software v4.0 (Affymetrix).

Antioxidant activity assays

Hypothalamus and cell samples were harvested and then 20 µg total protein were subjected to the TXN and TXNRD activity assessment by TXN activity fluorescent assay kit (IMCO, Sweden) and TXNRD assay kit (Sigma, MO, USA) according to the manufacturer's instructions. TXN activity was determined by the reduction of insulin disulfides by reduced TXN with TXNRD and NADPH as the electron donor. Excess TXNRD and NADPH were provided to keep TXN at reduced state. During this reaction, fluorescence intensity at 515-525 nm will be altered from low to high level. Different doses of human thioredoxin 1 (hTXN-1) were used to generate standard curve by calculating the rate of reaction (Δ fluorescence per minute). TXN activity of the sample were determined using the formula given by the standard curve. The TXNRD activity was assessed using a colorimetric assay which is based on the reduction of 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) with NADPH to 5-thio-2-nitrobenzoic acid (TNB), which is measured at 412 nm. TXNRD activity of samples was determined by measuring the increase in absorption at 412 nm. One unit of mammalian TXNRD in this experiment will cause an increase in A412 of 1.0 per minute per ml at pH 7.0 at 25 °C (Sigma).

Western Blot

Described in Chapter 2.

Statistical analysis

Described in Chapter 2.

Results

The expression of TXNIP was downregulated on knocking out SELENOM. To further investigate genes and signaling pathways affected by SELENOM deficiency, a gene expression study was conducted using microarray assay on mHypoE-44 cells and hypothalamic tissue samples from 10 week-old mice. Total RNA from the hypothalamic tissue (n=4) and mHypoE-44 cells (n=3) was isolated and then subjected to microarray assay using Clariom S Array Kit. Transcriptome Analysis Console software v3.0 was used to analyze differential gene expression profiles. In order to determine the differentially expressed genes (DEGs), we set two filter criteria: a fold change of greater than 50% and p-value <0.01 between the wild-

type and *Selenom*^{-/-} groups (Fig. 4-1). There are 1983 DEGs in mHypoE-44 cells and 167 DEGs in the hypothalamus which passed the filter criteria. Among those DEGs, 11 transcripts were significantly altered in both hypothalamic tissue and mHypoE-44 cells (Fig. 4-2A). One transcript of particular interest was the TXNIP, a known negative regulator of TXN and mammalian target of rapamycin (mTOR)-dependent signaling [179]. TXNIP expression was significantly decreased by SELENOM deficiency in both hypothalamic tissue and mHypoE-44 cells, which was verified by Western blot analysis (Fig. 4-2B-E). The results revealed that SELENOM deficiency induced a significant downregulation of TXNIP level, which could be a compensatory response to SELENOM ablation.

SELENOM deficiency significantly diminishes TXN activity. TXNIP, an endogenous inhibitor of TXN, plays an important role in the regulation of the TXN system, which consists of TXN, TXNRD, and NADPH. This major antioxidant system protects cells from oxidative stress through its thiol-reducing functions [179]. As described above, TXNIP is downregulated by knocking out SELENOM in both hypothalamic tissue and cell line. Thus, to further determine the effect of SELENOM in regulating TXN system activity, both TXN and TXNRD activity assays were performed on hypothalamic tissue and mHypoE-44 cells. In hypothalamic tissues, TXN activity was significantly decreased with no differences in TXNRD activity (Fig. 4-3A, B). However, in mHypoE-44 cells, SELENOM deficiency impeded TXN activity and TXNRD activity at the same time (Fig. 4-3C, D). The reason why SELENOM deficiency would impact TXNRD activity in cell culture but not hypothalamic tissue is still unknown. It could be related to the differences between an actual brain structure and an immortalized cell line. The results support the notion that SELENOM regulates the hypothalamic TXN system.

Discussion

To further elucidate the underlying mechanism of how SELENOM regulates leptin signaling, a microarray analysis was conducted in hypothalamus and mHypoE-44 cells to identify SELENOM-related genes and pathways. Our analyses identified 167 DEGs in hypothalamic tissue and 1983 DEGs in mHypoE-44 cells when filter criteria was set at a fold change of greater than 50% and p-value <0.01 between control and *Selenom*^{-/-} samples (Figure 4-1B). The hypothalamus is a heterogeneous brain

structure that contains 34 different neuronal clusters and 11 non-neuronal clusters [180]. This diversity of cell types present in the hypothalamus may largely contribute to the smaller number of DEGs observed in hypothalamic tissue. Among these 11 DEGs, we found that TXNIP was notably downregulated by SELENOM deficiency. The TXN system, consisting of TXN, TXNRD, and NADPH, is a key antioxidant system which controls cellular redox homeostasis. TXNs contain a redox active site (CXXC) and are kept in reduced form by TXNRDs using electrons transferred from NADPH. Disturbances of the TXN system have been associated with a wide range of health problems, including cancers, neurodegeneration, cardiovascular diseases, and metabolic disorders [181, 182]. One major regulatory influence upon this antioxidant system is TXNIP, an endogenous negative regulator of TXN [183]. Oxidized TXNIP binds to the reduced TXNs through a disulfide exchange, causing TXNs inactivation. TXNIP has emerged as a critical component in regulating cellular redox balance, particularly in relation to metabolic syndrome [184, 185]. Its level can be upregulated by ER stress via PERK and IRE1 α signaling pathways in β cells, leading to activation of interleukin-1 β production by the NLRP3 inflammasome [186-188]. This process is the main determinant of ER stress-mediated β cell death. Hypothalamic TXNIP also plays a critical role in nutrient sensing and energy metabolism, as downregulation of TXNIP protects against a HFD [184]. It was further demonstrated that conditionally overexpressing TXNIP in AGRP neurons leads to a reduction of energy expenditure and HFD-induced obesity, whereas TXNIP deficiency promotes leptin sensitivity [185]. In addition to TXNIP, TXN, and TXNRD also play significant roles in lipid homeostasis and energy metabolism [189-192]. For example, Rajalin et al. showed that both TXN and TXNRD activities were increased during the adipocyte differentiation, whereas inhibitors of adipogenesis decreased TXNRD activity [193]. In the present study, TXNIP was downregulated by knocking out SELENOM, indicative of a compensatory response to increase TXN activity and re-establish cellular redox homeostasis.

Among all the DEGs in hypothalamic tissue, TXNIP is the only gene of which protein levels were confirmed to decrease in *Selenom*^{-/-} samples. However, there are some other genes of potential interest due to their specific functions involved in energy metabolism (Table 3). The CCAAT/enhancer binding protein delta (Cebpd) is a transcription factor that regulates many biological processes including cell differentiation, proliferation, and cell death. Cebpd was found to affect adiposity and body weight in female animals. Additionally, female *Cebpd*^{+/-} animals also showed a decrease in circulating insulin level

with no effect on leptin levels. However, leptin levels are reduced in *Cebpb^{-/-}* mice, indicating that *Cebpd* is also associated with leptin regulation [194]. Lipocalin-2 (*Lcn2*), also known as neutrophil gelatinase-associated lipocalin (NGAL), is well characterized as a mediator of inflammatory processes [195]. It is a major regulator in intestinal inflammation and in turn, regulates the intestinal metabolic immune interactions. In adipose tissue, increased *Lcn2* levels were found in *ob/ob* mice, *db/db* mice, and mice with HFD [196]. Moreover, it is associated with insulin resistance in hepatocytes and β cells via regulation of inflammatory responses [197, 198]. Another DEG, cyclin dependent kinase inhibitor 1A (*Cdkn1a*), is a cyclin-dependent kinase inhibitor that acts as a regulator of cell cycle progression at G1 and S phase via its inhibitory function on activity of cyclin-cyclin-dependent kinases. Expression of *Cdkn1a* is regulated by the tumor suppressor protein 53 (p53) and appears to play a critical role in the DNA damage and cell cycle. These candidates give us some potential targets to explore the biological functions of SELENOM in the future.

Taken together, our findings conclusively demonstrate that the hypothalamic TXN system is regulated by SELENOM and that this potentially contributes to hypothalamic leptin signaling. Moreover, besides TXNIP, several additional DEGs were identified by our microarray studies, and these include genes involved in energy homeostasis, inflammation, and cell cycle. These results provided us with several putative pathways affected by SELENOM deletion and offer valuable directions for our future studies in SELENOM.

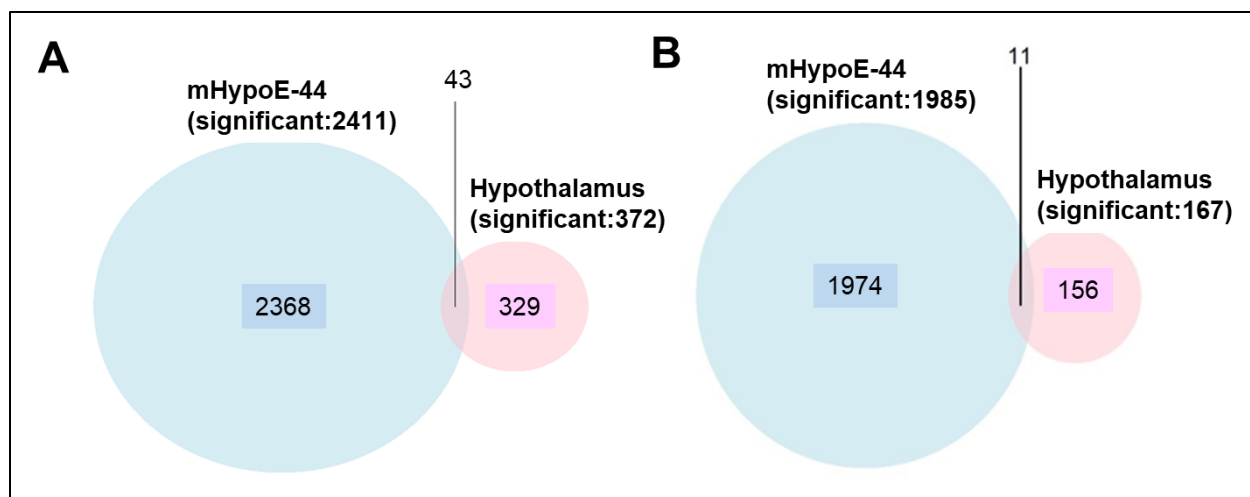


Figure 4-1. Venn diagrams showing the number of DEGs between control and *Selenom*^{-/-} samples.

Filter criteria was set as fold change of greater than 50% and a p-value<0.05 (**A**) or p-value<0.01(**B**).

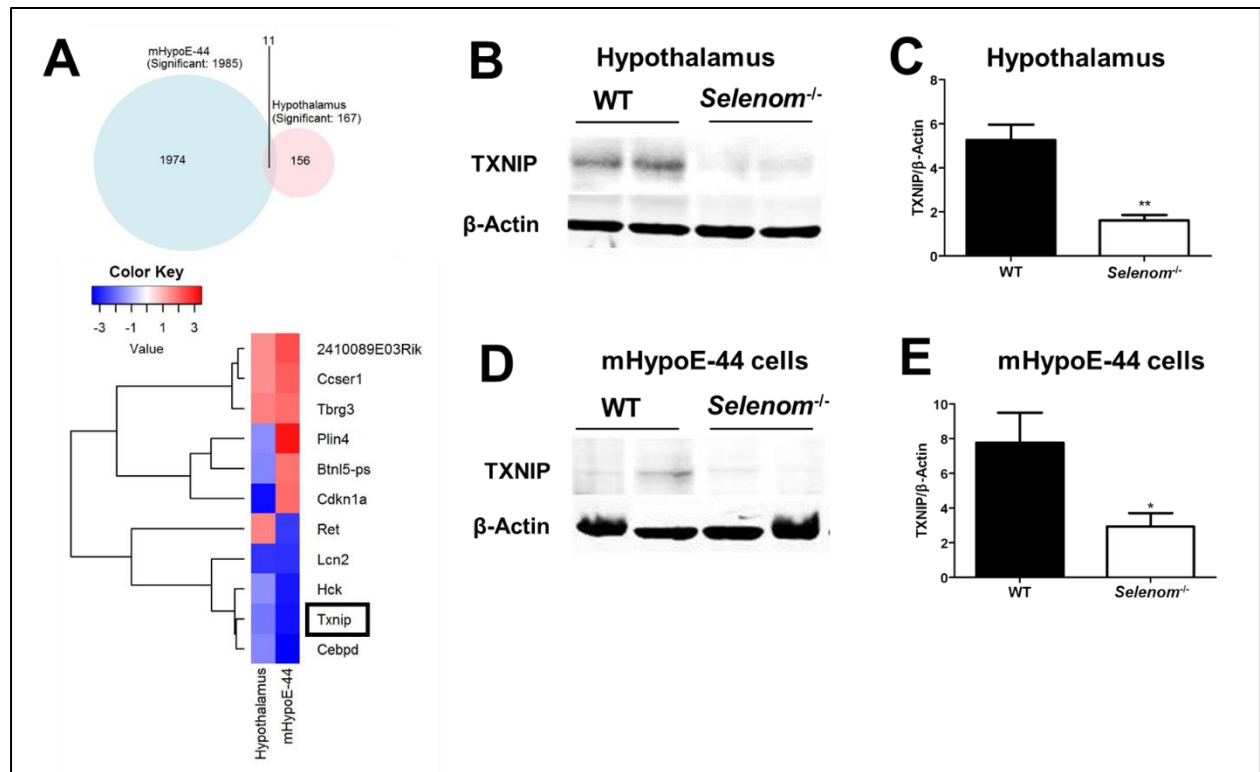


Figure 4-2. The expression of TXNIP was downregulated by knocking out SELENOM. **A**, Venn diagram (top) showing the number of DEGs determined by the microarray analysis on mHypoE-44 cells and hypothalamic tissue. Transcripts showing a fold change of greater than 50% and p-value <0.01 between control and *Selenom*^{-/-} samples were considered to be DEGs. 11 DEGs were affected in both hypothalamic tissue and mHypoE-44 cells. Heatmap plot (bottom) of these 11 DEGs, with upregulation and downregulation of fold change represented by red and blue, respectively. **B**, **D**, Western blot analysis of TXNIP protein levels in hypothalamic tissue (**B**) and mHypoE-44 cells (**D**). **C**, **E**, TXNIP protein levels were significantly diminished by SELENOM deficiency in both hypothalamic tissue (**C**) and mHypoE-44 cells (**E**). Results shown represent the means ± SEM (unpaired Student's t-tests) for at least three independent experiments. *p<0.05, **p<0.01 compared to control group.

Table 3. Summary of Differentially Expressed Genes Affected by SELENOM Deficiency					
Gene	Hypothalamus		mHypoE-44		Functions
	Fold change	P value	Fold change	P value	
Cdkn1a	-3.29	0.0014	1.99	0.0013	Cell cycle [199]
Lcn2	-2.76	0.0010	-2.82	3.14E-05	NF- κ B pathway, Inflammation [200]
TXNIP	-1.84	0.0002	-3.22	4.65E-06	Oxidative stress Inflammation [186],
Cebpd	-1.64	0.0005	-3.46	3.63E-07	Inflammation [201], Adipocyte differentiation [202]
Btl5-ps	-1.64	0.008	1.89	0.0003	NF- κ B signaling [203]
Plin4	-1.56	0.0039	3.19	8.77E-06	Lipid accumulation [204]
Hck	-1.52	0.003	-3.12	6.94E-05	Inflammation [205]
Tbrg3	1.71	0.0017	1.97	0.0008	Unknown
Ret	1.66	0.0022	-2.68	0.0003	Cancer [206]
Ccser1	1.53	0.0036	2.17	0.0001	Unknown
2410089E03Rik	1.52	0.0096	2.41	1.43E-05	Unknown

Table 3. Summary of differentially expressed genes affected by SELENOM deficiency. Transcripts showing a fold change of greater than 50% and p-value <0.01 between control and *Selenom*^{-/-} samples were considered to be DEGs. 11 DEGs were affected in both hypothalamic tissue and mHypoE-44 cells, with upregulation and downregulation of fold change represented by red and green.

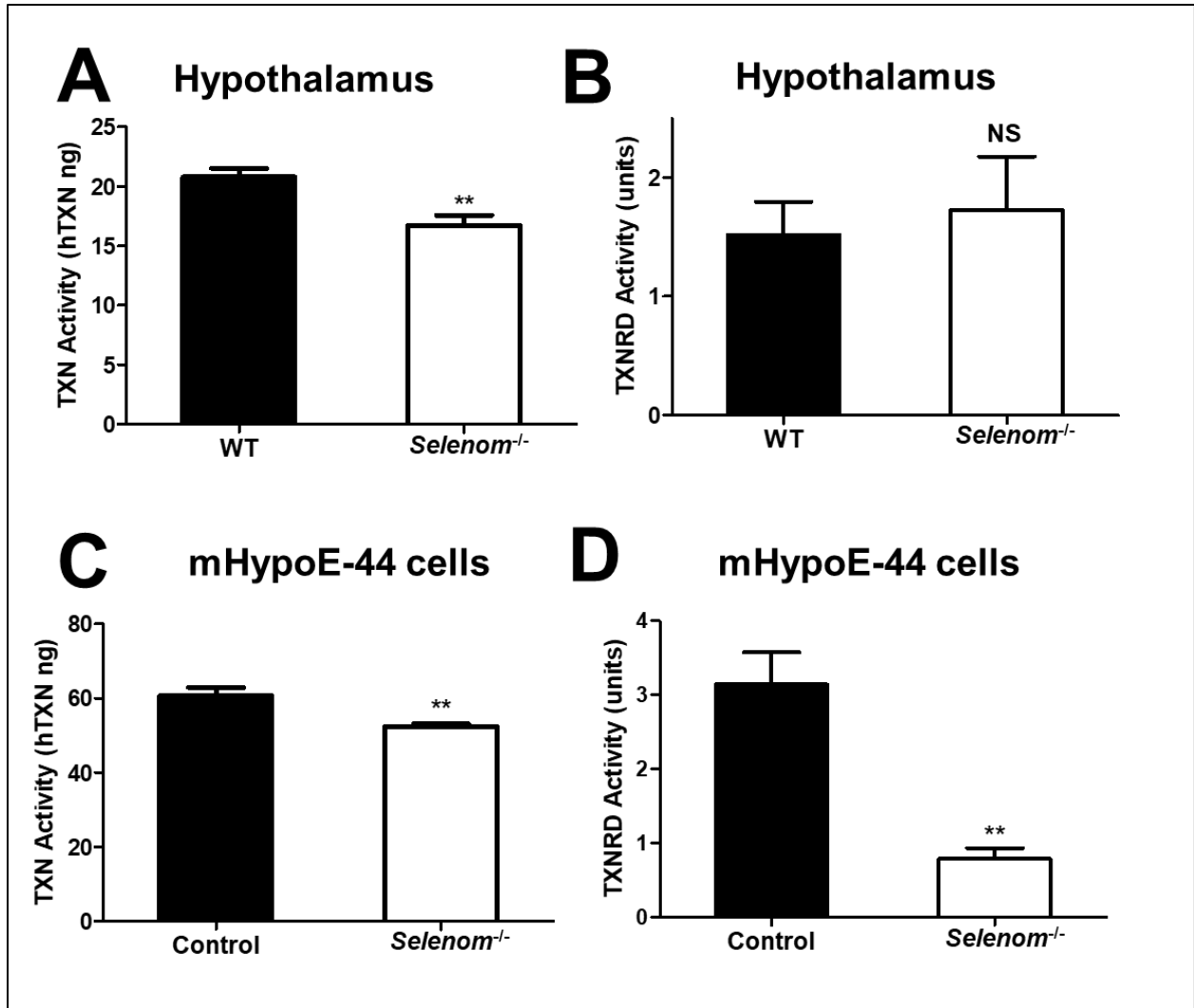


Figure 4-3. SELENOM deficiency significantly impacts TXN activity in hypothalamic tissue and mHypoE-44 cells. **A, C,** Levels of TXN activity were significantly reduced by SELENOM deficiency in both hypothalamic tissue (**A**) and mHypoE-44 cells (**C**). **B, D,** Levels of TXNRD activity were unaffected by SELENOM deficiency in hypothalamic tissue (**B**), but were significantly diminished in *Selenom*^{-/-} samples in mHypoE-44 cells (**D**). Results shown represent the means \pm SEM (unpaired Student's t-tests) for at least three independent experiments. ** $p < 0.01$ compared to control group.

CHAPTER 5 CONCLUSION

Summary of the Findings

We found that SELENOM expression in wild-type hypothalamic tissue was increased in response to leptin treatment, indicating SELENOM is a leptin-responsive gene. *Selenom*^{-/-} mice displayed impaired leptin sensitivity, as leptin-induced Stat3 phosphorylation was largely reduced compared to wild-type mice. Similar results were found in hypothalamic mHypoE-44 cells. Moreover, leptin treatment led to a reduction of baseline cytosolic Ca²⁺ in the control group, and this effect was attenuated by knocking out or knocking down SELENOM. We then assessed the effects of SELENOM on ER stress, a well-known cause of leptin resistance. In response to thapsigargin, increased ER stress was found in SELENOM-deficient mHypoE-44 cells compared to the control group, indicating that SELENOM suppresses hypothalamic ER stress. Additionally, thapsigargin also induced a robust increase in cytosolic Ca²⁺ when SELENOM was knocked down or deleted. Furthermore, our findings add novel evidence that SELENOM is a positive modulator of the TXN system in the hypothalamus, which can potentially contribute to hypothalamic leptin signaling. Microarray analysis on hypothalamic tissues and cells revealed one DEG of particular interest, TXNIP, a known negative regulator of TXN. In addition, TXN activity was significantly decreased by SELENOM deficiency. Lastly, we assessed SELENOM subcellular localization and identified two novel SELENOM interaction partners, MAP6 and PCYT1A. Results from subcellular fractionation determined that SELENOM also resides in MAMs and the mitochondria, in addition to the ER.

Thus, our findings conclusively delineate the positive role of SELENOM in leptin signaling and TXN activity in hypothalamus (Fig. 5-1). Moreover, our results revealing new subcellular localizations and interacting partners of SELENOM point to new directions for investigating in the biological functions of SELENOM.

General Discussion and Future Directions

One of the main findings of this work is that SELENOM deficiency impairs leptin signaling. However, in our microarray studies, we did not find any changes in the mRNA levels of the two best established negative regulators of leptin signaling, SOCS3 and PTP1B. The decreased leptin sensitivity in *Selenom*^{-/-} samples may be due to other factors. Interestingly, among 11 DEGs from the microarray assay, 4 transcripts, implicated in inflammation, including Lcn2, TXNIP, Cebpd, and Hck, were significantly downregulated in *Selenom*^{-/-} samples. Multiple studies have revealed that activation of proinflammatory signaling pathways in hypothalamus promotes leptin resistance [124, 207-209]. Lcn2 is of particular interest in regulating the inflammation pathways, especially NF- κ B pathways. NF- κ B is a transcription factor that regulates inflammatory signaling pathways. Activation of hypothalamic NF- κ B-mediated signaling inhibits Stat3 activation, leading to impaired leptin signaling, and increased expression of interleukin 1 (IL-1), IL-6, and TNF α [207, 210]. Lcn2 has been characterized as an anti-inflammatory cytokine/adipokine in obesity and inflammation [198]. Lcn2 deficiency results in increased activation of NF- κ B signaling induced by lipopolysaccharides (LPS) [211]. In addition, Lcn2 has been reported to suppress inflammation via inhibition of the NF- κ B pathway in retinal degeneration and uveitis [212, 213]. We speculate that SELENOM deficiency may activate the NF- κ B pathway and inhibit Stat3 activation, leading to impaired leptin signaling. However, questions still remain as to whether and how SELENOM deficiency affects the hypothalamic immune system via Lcn2. Future experiments will determine changes and functions of proinflammatory cytokines in *Selenom*^{-/-} hypothalamic tissue and how these cytokines contribute to leptin signaling.

Mitochondria have an essential role in ROS generation and mitochondrial bioenergetics is largely determined by Ca²⁺ signaling via MAMs. These ER-mitochondria contact sites are rich in proteins involved in ER-mitochondria tethering, phospholipid metabolism, Ca²⁺ signaling, and protein folding. Intriguingly, multiple ER chaperones that localize to MAMs are TXN family members or selenoproteins. Endoplasmic reticulum resident protein 44 (ERp44), containing a TXN domain, interacts with IP3R1 and competes with binding immunoglobulin protein (BiP)/Grp78 for the binding sites [98, 99]. ERp44 overexpression reduced IP3R1-mediated Ca²⁺ release and in turn, prevented Ca²⁺-induced apoptosis via IP3R1 [98]. Another two TXN family members, TXN-related transmembrane protein 1 (TMX1) and endoplasmic reticulum DNA J domain-containing protein 5 (ERdj5), both act on SERCA2b but have opposite functions. TMX1 is enriched in the MAMs and exhibits an inhibitory function on SERCA2b. TMX1 promotes Ca²⁺ flux from the

ER to mitochondria upon binding to and inactivating this Ca^{2+} uptake channel, therefore affecting mitochondrial bioenergetics [214, 215]. ERdj5 also contains TXN domains and acts as a reductase in the ER. It activates SERCA2b under stressed conditions, thereby maintaining ER Ca^{2+} levels and protecting cells from mitochondrial dysfunction [216]. Like ERdj5, SELENOM can reduce luminal cysteines of SERCA2b and then activate this Ca^{2+} channel [101]. In our study, SELENOM, a member of the TXN superfamily, was found to localize in MAMs and constitutes an important component of the hypothalamic TXN system. Additionally, we have promising data showing that SELENOM deficiency promotes Ca^{2+} release from the ER in hypothalamic cells in response to Tg-induced ER stress. MAMs are dynamic structures that will change their key parameters, size of the area and the gap width between the ER and the mitochondria in response to various disturbances. We speculate that SELENOM deficiency will help to expand MAMs and promote the Ca^{2+} flux under a stressed state. Moreover, we have observed that IP3R1 and RyR3 are co-expressed with SELENOM in the mouse brain and may be affected by SELENOM deficiency. Therefore, SELENOM-mediated increase of Ca^{2+} flux to the mitochondria may also potentially result from its interaction with Ca^{2+} channels or pumps, such as SERCA and IP3R. This process may subsequently regulate the function of mitochondria, including ATP generation, ROS generation, and apoptosis signaling. However, the present study did not determine: 1) the subcellular compartment where cytosolic calcium is pumped in response to leptin in mHypoE-44 cells; 2) whether SELENOM affects mitochondrial calcium levels; or 3) whether SELENOM influences the morphology of MAMs. Further experiments are necessary to decipher the influence of SELENOM at MAMs with respect to Ca^{2+} signaling, and mitochondria function.

Meanwhile, we observed that SELENOM interacts with MAP6 and PCYT1A, two key proteins implicated in microtubule stabilization and phosphatidylcholine biosynthesis, respectively, indicating that SELENOM may be involved in modulation of ER-related membrane biosynthesis. As mentioned above, PCYT1A is the rate-limiting enzyme in PC synthesis and its activity is increased upon translocation to the ER [173]. Moreover, PC depletion induced by inactivation of PCYT1A increases the expression of CHOP, indicating that PCYT1A deficiency may induce an ER stress response and influence ER membrane biosynthesis [144]. We propose that SELENOM binds to and stabilizes PCYT1A in the ER to promote its function, whereas SELENOM deficiency will induce PC depletion via inactivation of PCYT1A. This is

consistent with our current findings that the expression of CHOP was increased when knocking down SELENOM. However, questions still remain as to the mechanisms by which SELENOM influences the functions of these two interacting partners in biological processes. Further investigation of SELENOM will be necessary to delineate the specific relationship between SELENOM and of these two interaction partners in the context of oxidative stress.

In addition to energy metabolism, SELENOM has also been implicated in the field of cancer. Selenium treatment in the SELENOM overexpression transgenic rat model was reported to activate the MAPK pathway [94]. Consequentially, hyperactivation of the MAPK pathway has also been reported in hepatocellular carcinoma (HCC). Based on this possible correlation between SELENOM and HCC, Guariniello et al. evaluated the SELENOM expression in two HCC cell lines, HepG2 and Huh7, compared to the normal hepatocytes. They found an elevated level of SELENOM in HCC cell lines by real-time PCR and Western Blot, suggesting the involvement of SELENOM in HCC [79]. Further investigation confirmed this finding in tumor samples from patients with HCC by immunohistochemistry, which suggests that SELENOM expression in liver tissues of HCC patients could be a potential prognostic tool for understanding HCC progression [217]. Herein, we have determined that SELENOM is a positive regulator of TXN system. The TXN system plays a critical role in the regulation of cell growth and cell survival. One established point of regulation is the inhibition of MAPK signaling mediated by the binding of reduced TXN1 to apoptosis signal-regulating kinase 1 (ASK-1) [218]. ASK-1 is an upstream regulator of MAPK signaling which is critical in oxidative stress-induced apoptosis [219]. In addition, TXN1 levels are increased in many human cancers [220-222]. Cancer cells use TXNs as protectors against oxidative stress-induced apoptosis. This may at least partially explain why SELENOM is highly expressed in HCC compared to normal cells. Since SELENOM is a positive modulator of TXN system, it may be also a potential target for cancer therapy. Further investigation is necessary to delineate this novel redox signaling mediated by SELENOM and its functions in cancers and other redox imbalance-related disorders.

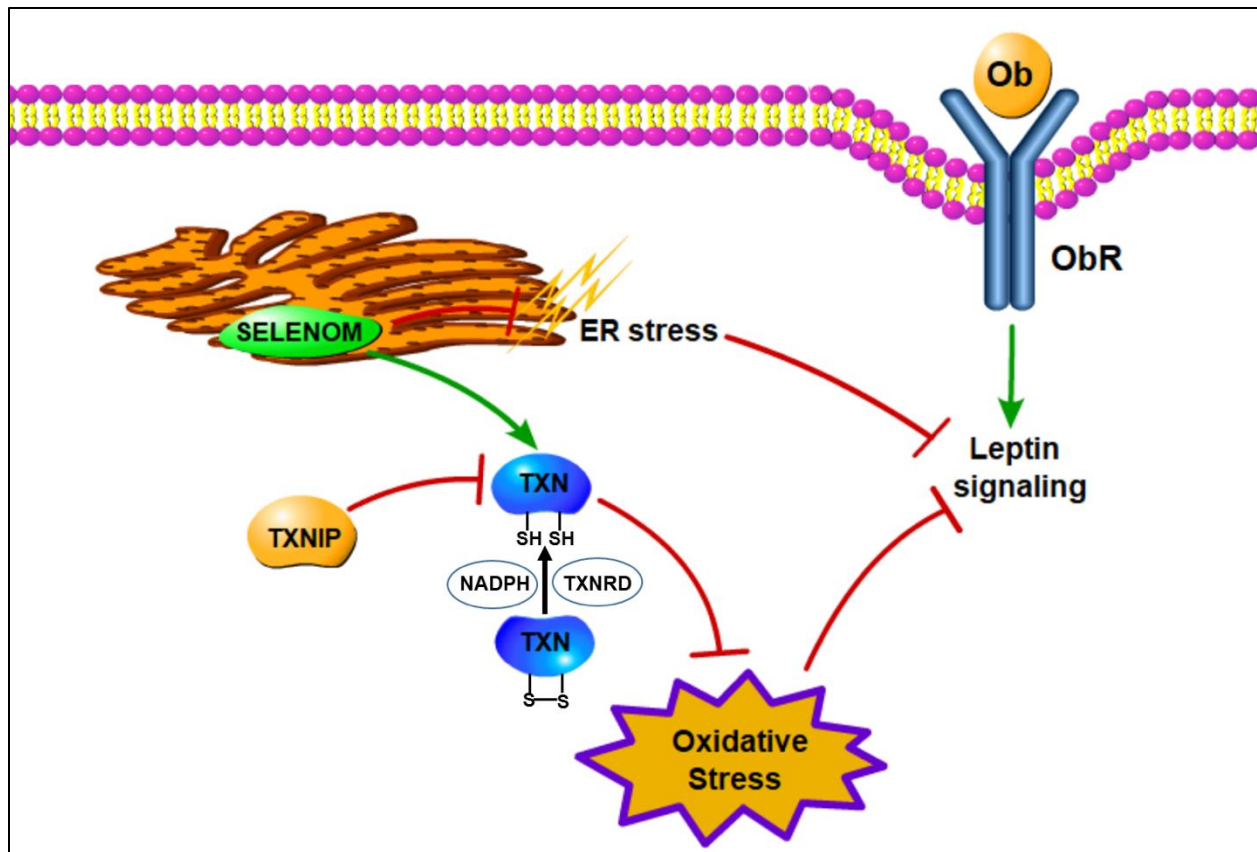


Figure 5-1. Hypothetical model of SELENOM function. SELENOM is an ER-resident oxidoreductase which is an important component of the hypothalamic TXN system. Genetic ablation of SELENOM disturbs ER homeostasis and impedes leptin signaling, leading to impairments in energy metabolism.

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